Expression of growth factors and growth factor receptors in human cleft-affected tissue

Benita Krivicka, Mara Pilmane, Ilze Akota

SUMMARY

Objective. To investigate cleft disordered tissue in children with cleft palate and cleft lip with or without alveolar clefting for detection of local tissue growth factors and growth factor receptors and compare findings.

Design. Morphological analysis of human tissue.

Patients. Three groups were studied: 14 patients with cleft palate at the age from eight months to 18 years and two months, 12 patients with cleft lip with or without alveolar clefting in the age from four months to 15 years and four months and 11 control patients.

Results. In general, cleft palate disordered tissue showed more prominent expression of BMP2/4 (z=3.574; p=0.0004) and TGFβ (z=2.127; p=0.033), while expression of TGFBR3 significantly higher was only in connective tissue (z=3.822; p=0.0001). Cleft lip affected tissue showed significantly pronounced expression of FGFR1 in general as well as separately in epithelium.

Conclusions. The marked and statistically significant expression of BMP 2/4 in cleft palate disordered soft tissue probably is delayed, but still proliferation and differentiation as well as tissue, especially, bone remodeling contributing signal. Cleft palate affected tissue show more prominent expression of TGFβ, still the weak regional expression of TGFβ type III receptors prove the disordered tissue growth and changed TGFβ signalling pathway in postnatal pathogenesis. In general, expression of TGFβ, BMP 2/4 and FGFR1 is significantly different, giving evidence to the involvement of these mentioned factors in the cleft severity morphopathogenesis.

Key words: TGFβ, TGFBR3, BMP2/4, bFGF, FGFR1.

INTRODUCTION

Clefts of the lip and palate are among the most common birth defects worldwide. In Latvia the cleft lip and cleft palate occur on the average in one child in 700-800 newborn infants, which is the second widely met pathology in embryos and fetus (1, 2).

Development of the face and jaws is the product of growth and fusion of five facial prominences and involves cell migration, proliferation, differentiation and apoptosis. These primordiae consist of a mesen-chymal core derived mainly from the cranial neural crest cells and of an ectodermally derived epithelial outer covering (3). One of more complicated event is development of secondary palate. The main part of secondary palate is formed by two shelf-like outgrowths from the maxillary prominences. Mentioned palatal primordiae are covered by medial edge epithelium (MEE). After fusion of palatal shelves MEE adhere forming epithelial midline edge seam (MES), what is one of the more investigated moments in the development of secondary palate (4). Moreover, the exact fate of the epithelia in the MES is still controversial, and three major pathways have been proposed for their disappearance: apoptosis, migration to the oral or nasal side of the palate, and epithelial-mesenchymal transformation (5-7). That is also still emphasided in the review article of Iseki (8). All mentioned processes in the embryonic tissues are caused and regulated by various factors, especially some growth factors and growth factor

[1]*Institute of Anatomy and Anthropology, Riga Stradins University, Riga, Latvia*
[2]*Department of Oral and Maxillofacial Surgery, Institute of Stomatology, Riga Stradins University, Riga, Latvia*

Benita Krivicka[1] *– PhD.*
Mara Pilmane[1] *– PhD., Dr. hab. med., prof.*
Ilze Akota[2] *– D.D.S., MSc (Oslo), PhD., prof.*

Address correspondence to Dr. Benita Krivicka, Institute of Anatomy and Anthropology of Riga Stradins University, Dzirciema street 16, Riga, LV-1007, Latvia.
E-mail address: b.krivicka@inbox.lv

receptors, whose study has been intensified within the recent two ten years and is still actively going on.

The most studied secreted proteins in palatal development belong to the TGFβ superfamily. This is a group of structurally related growth and differentiation factors, including TGFβs and bone morphogenetic proteins (BMP). Mentioned growth factors are involved in many biological processes that also occur during palatogenesis, such as cell proliferation, apoptosis, extracellular matrix synthesis and deposition, cell migration, epithelial-mesenchymal transformation, and degradation of basement membrane (7, 9).

TGFβ plays important role during various stages of palate development (9). It is an extracellular protein predominantly produced by a subset of T cells and is ubiquitously expressed by all cells. In mammals, three isoforms of TGFβ are currently identified. All three (TGFβ-1, TGFβ-2, TGFβ-3) of these proteins share extensive regions of similarity in their amino acids (10). The role of various TGFβ isoforms in palate development is still under investigations. Irrespective of variation of views among researchers about the exact timing and localization of TGFβ isoforms in palatogenesis, most agree that TGFβ-1 plays a crucial role in cell proliferation and growth of the shelves. TGFβ-1 and TGF-2 regulate mesenchymal cell proliferation and extracellular matrix synthesis in the palate, whereas TGFβ-3 orchestrates fusion of the palatal seam (7, 9). In the TGF-β3 null-mutant mice the palatal shelves fail to adhere properly, the basement membrane is not degraded and the MEE do no disappear from the midline seem (11).

TGFβs exert its effect by interacting mainly with three membrane proteins named type I (RI), type II (RII) and type III (RIII) receptors (12). Both type I and type II receptors are transmembrane serine/threonine kinases indispensable for TGFβ signalling. Type III receptor, also termed betaglycan, is a membrane-anchored protein lacking a cytoplasmatic kinase domain, and serves as a direct modulator of TGFβ access to the signalling receptor. In the presence of activated TGFβ ligands, TβR-III forms a transient heteromeric complex with TβR-II and presents TGFβ directly to TβR-II (13). In co-receptor role TβRIII directly binds ligands in the TGFβ superfamily, including BMP-2 and BMP-4 (14).

TβR-III has been shown is specifically expressed in the medial edge epithelium in a distribution similar to TGFβ3 at critical stages of palatal shelf adherence and has a critical role in the palatal fusion (11). However, investigations show, that after palatal shelves fusion mesenchymal cells continue to express TGFβ3, however TβR-III was only expressed in epithelium (15). Scientists from USA report, that TβRIII is ubiquitously expressed on nearly all cell types and is the mostly highly expressed of the TGFβ superfamily receptors on those cells, with at least 200.000 receptors/cell as opposite to 5000-10.000 receptors/cell for most TGFβ superfamily type I and type II receptors (14).

The bone morphogenetic proteins (BMPs) as mentioned early, belong to the TGFβ superfamily and are implicated in the mammalian palate development (16). BMP2 and BMP4 are expressed in mammalian palate both the epithelia and mesenchyme prior to, during and after palatal shelf fusion, but mainly act as mesenchymal proliferation contributing factors (17). Mentioned growth factor signals are very important for closure of the upper lip or primary palate (18).

The fibroblast growth factors are major regulators in embryonic development. The 22 members of the FGF family mediate their cellular responses by binding to and activating the different isoforms encoded by the four receptor tyrosine kinases designated FGFR1, FGFR2, FGFR3 and FGFR4 (19). These growth factors have emerged as a key contributors in epithelial – mesenchymal dialogue contributing to morphogenesis of the orofacial region and are involved in almost all structures development (20). Its known, that basic FGF influences collagen synthesis and extracellular matrix proteins at the time of mammalian palatal fusion, but FGFR1 is expressed specifically in the epithelium of the developing palatal shelves from the time of the outgrowth from the maxillary process through completion of fusion (21).

Nonetheless, irrespective of many studies, the data on distribution and localization of various growth factors and growth factor receptors in human cleft-affected tissues are still not clear. Therefore in this study we focused on the relative abundance and localization of growth factors and growth factor receptors in children cleft palate and cleft lip-affected tissue.

The aim of our study was to investigate cleft disordered tissue in children with cleft palate and cleft lip for detection of local tissue growth factors and growth factor receptors, to cross-correlate the obtained data.

MATERIALS AND METHODS

The research is based on the material of cleft lip and cleft palate patients, which was gathered within

a period of time from 2003 to 2006 at the Cleft Lip and Palate Centre of the Institute of Stomatology of the Riga Stradins University. Permission of the Ethics Committee: decision of the RSU Ethics Committee dated 22nd May 2003.

The research involved 14 patients with cleft palate at the age from eight months to 18 years and two months and 12 patients with cleft lip with or without alveolar clefting in the age from four months to 15 years and four months.

The control material was obtained from 11 patients with oral-facial trauma and cleft-unrelated surgical operations. The material was gathered at the Institute of Stomatology of the Riga Stradins University within a period of time from 2004 to 2006.

Immunohistochemistry method (Hsu *et al.*, 1981). Tissues were fixed for a day in mixture of 2% formaldehide and 0.2% picric acid in 0.1 M phosfate buffer (pH 7.2). After samples were rinsed in thyroide buffer, containing 10% sacharose for 12 hours, then embedded into paraffin and cut in 6-7 µm thin sections. Sections were proceeded for detection of following growth factors and growth factor receptors: transforming growth factor beta (TGFβ) (code ab 1279, work dilution 1:1000, Abcam, UK), transforming growth factor beta receptor type III (TGFBRIII) (code LS-B3392, work dilution 1:100, LsBio); bone morphogenetic protein 2/4 (BMP 2/4) (code AF355, work dilution 1:100, RnDSystems, Germany), basic fibroblast growth factor (bFGF) (code ab 16828, work dilution 1:200, Abcam, UK), fibroblast growth factor receptor 1 (FGFR1) (code ab 10646, work dilution 1:100, Abcam, UK).

Routine histological staining with haemotoxylin and eosin was developed for each case to get review picture of the slide.

Distribution of immunoreactive structures was detected semiquantitatively (22, 23). The quantity of structures was analyzed in five visual fields of one section. Explanatory notes on the applied markings are given in Table 1.

Statistical analysis. We used the Mann-Whitney test for comparison of two independent groups, applying the ranking values. Values of $p < 0.05$ were considered significant. For comparison of a number of samples we used the Kruskal Wallis test.

The correlation coefficient r as a quantitative indicator of coherence tightness between two or more variables calculated for the ranking values (Spearman's Rank Correlation Coefficient). In the study the qualitative coherence tightness between variables, on the grounds of the correlation coefficient value, was assessed as weak, average or tight. The distribution of the correlation coefficient was as follows: $r=0-0.3$, low, insignificant correlation; $r=0.4-0.7$, average correlation; $r=0.7-0.9$, tight correlation. A statistical analysis was carried out by means of the SPSS (SPSS Inc., USA).

RESULTS

TGFβ

Control material showed expression of TGFβ almost in all cases. Immunoreactive epithelial cells were found in the middle layers of stratified squamous mucosal epithelium and relative amount mainly varied from moderate to numerous, but on average was moderate (Table 2). Underlaying connective tissue more frequently contained few or rare positive connective tissue cells among them inflammatory cells. Relative amount of TGFβ containing endothelial cells mainly varied from few to moderate, but immunoreactivity of vascular smooth muscle cells was less pronounced.

TGFβ immunoreactive epithelial and connective tissue cells we observed in 12 cleft palate affected tissue. The relative number of positive epitheliocytes was very variable. Mentioned cells mainly localized in the middle layers of stratified squamous mucosal epithelium and frequently were vacuolized. Expression of TGFβ in connective tissue was slight and varied from rare to moderate. TGFβ positive endothelial cells mainly in the walls of sclerotic blood vessels and vascular smooth muscle cells we observed in all cleft palate affected tissues.

In general, expression of TGFβ in cleft lip affected tissue was less pronounced. Mainly we found

Table 1. Marking of relative frequency of the immunohistochemically determined structures

Applied markings	Explanatory notes
-	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
+++/++++	Plenty of positive structures in the visual field

moderate number of positive mucosal epithelial cells. Immunoreactive epidermal cells as well as cells in sebaceous glands and hair follicles were detected only in rare cases. The relative number of TGFβ containing connective tissue cells was very variable. Mentioned growth factor also marked variable number of endothelial and smooth muscle cells in the walls of blood vessels. Moreover, the relative number of positive structures exactly in the walls of blood vessels was statistically significantly less than in cleft palate disordered tissue ($z=2.455$; $p=0.014$).

Moreover, as well as in general Mann-Whitney showed statistically significant differences between relative number of TGFβ containing structures in cleft palate and cleft lip patients groups ($z=2.127$; $p=0.033$) (Table 3).

TGFBR3

Expression of TGFBR3 in epithelium and in the walls of blood vessels we didn't found in any control material. Mentioned receptors mainly marked rare or few connective tissue cells among them inflammatory cells.

Expression of TGFBR3 was not characteristic for cleft palate disordered epithelium except three cases. Relative number of immunoreactive connective tissue cells varied, but more often it was rare. The walls of blood vessels mainly demonstrated absence of immunoreactive structures, however, in four cases relative number of positive cells considerably varied.

Cleft lip affected tissue showed distinct expression of TGFBR3 in distribution of positive structures. We saw positive reaction in basal epithelial cells, in basal cells of hair follicle and serous cells of minor salivary gland. The immunoreactivity in connective tissue was not found in any case, and this difference was statistically significant, if compare with results with cleft palate affected tissue ($z=3.822$; $p=0.0001$). Similar as in cleft palate affected tissue, we found positive structures in the walls of blood vessels in four cases, but expression was slight. The relative number of positive structures statistically significantly differed in epithelium ($z=2.804$; $p=0.005$), connective tissue ($z=4.029$; $p=0.0001$) and in the walls of blood vessels ($z=1.965$; $p=0.049$),

Table 2. Semiquantitative distribution of immunoreactive structures in the material of children with cleft palate (1), in the material of children with cleft lip with or without alveolar clefting (2) and in control group patients (K)

	TGFß			TGFBRIII			BMP2/4			bFGF			FGFR1		
	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.
1	0-++- +++	0/+- ++	++	-	0/+- ++	-	++- +++	++/ +++	0-+- ++	-	0/+	-	+-++- +++	0/+	0-+- ++
2	+/++	0-++- +++	0/+- ++	0/+*	-*	0-+*	++	0-++*	0-++	0-0/ +-++	-*	-	++/ +++	+-++	++
K	++	+	+/++	-*	0/+*	-*	++	++*	+-++	-	0/+*	-	++	0/+-+	+

"-" – 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, "e." – epithelium, "c.t." – connective tissue, "b.v." – blood vessels.
* statistically significant diferences between cleft palate or cleft lip with or without alveolar clefting patients and control group.

Table 3. Semiquantitative distribution of immunoreactive structures in the material of children with cleft palate (1) and in the material of children with cleft lip with or without alveolar clefting (2)

	TGFß			TGFBRIII			BMP2/4			bFGF			FGFR1		
	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.	e.	c.t.	b.v.
1	0-++- +++	0/+- ++	++*	-	0/+- ++*	-	++- +++*	++/ +++*	0-+- ++	-	0/+*	-	+-++- +++*	0/+	0-+- ++
	($z=2.127$; $p=0.033$)*						($z=3.574$; $p=0.0004$)*						($z=2.853$; $p=0.0043$)*		
2	+/++	0-++- +++	0/+- ++*	0/+ ↑	-*	0-+	++*	0-++*	0-++	0-0/ +-++	-*	-	++/ +++*	+-++	++

"-" – 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, "e." – epithelium, "c.t." – connective tissue, "b.v." – blood vessels.
* statistically significant differences between cleft palate and cleft lip with or without alveolar clefting patients.

if compared to the control group's material. Moreover, Spearman's rank correlation showed weak significant correlation between TGFβ and TGFBRIII (p=0.045; r=0.205)

BMP 2/4

Expression of BMP 2/4 in control material was variable. The relative number of immunoreactive epithelial cells mostly varied from few (three cases) to moderate (five cases). Findings in connective tissue and in the walls of blood vessels also were similar and relative amount of positive cells mainly varied from few to moderate.

Cleft palate affected epithelium and connective tissue showed expression of BMP 2/4 in all cases. Relative amount of positive cells in both tissues mainly varied from moderate to many. BMP 2/4 marked variable number endotheliocytes and vascular smooth muscle cells in the nine tissue samples. Although expression of mentioned growth factor in epithelium and connective tissue was more pronounced, if compared with control, statistically significant differences were not stated.

Expression of BMP 2/4 in cleft lip disordered epithelium was variable. Mainly we found moderate immunoreactive cells as well as frequently BMP 2/4 expressed also cells of hair follicles and sebaceous glands. Moreover, we stated, that the difference between relative number of positive cells in cleft lip and in cleft palate affected epithelium is statistically significant (z=2.149; p=0.031). BMP 2/4 marked mainly moderate number of connective tissue cells in five tissue samples and also moderate number of endothelial and vascular smooth muscle cells in four cases. Exactly in connective tissue relative amount of positive structures was statistically significantly less, if compared with control (z=2.228; p=0.002) and with cleft palate tissue group (z=3.487; p=0.0005). Moreover, as well as in general Mann-Whitney showed statistically significant differences between relative number of BMP 2/4-containing structures in cleft palate and cleft lip patients groups (z=3.574; p=0.0004).

bFGF

Expression of bFGF in control material was slight and rare.

Expression of bFGF in cleft palate affected tissue generally was similar. We saw weak positive reaction in epithelium only in four cases. Slight expression of mentioned growth factor in connective tissue cells was found in nine tissue material. Basic FGF containing endothelial and vascular smooth muscle cells we found in three tissue samples.

Basic FGF stained variable number of cells of sebaceous glands in five cleft lip disordered tissue samples. Slight expression in connective tissue and vascular smooth muscle cells we found in rare cases. Mann-Whitney test demonstrated, that differences in relative abundance of positive connective tissue cells were statistically significant in comparing with control group (z=2.225; p=0.026) and also with cleft palate group (z=2.221; p=0.026).

FGFR1

Expression of FGFR1 in the control material was found almost in all tissue samples and more prominent and regional it was exactly in epithelium. FGFR1 mainly stained rare or few number of connective tissue cells as well as cells in the walls of blood vessels.

Cleft palate disordered tissue showed variable expression of FGFR1 in epithelium, connective tissue and in the walls of blood vessels. It was more prominent again in the epithelium.

Cleft lip affected tissue showed more prominent expression of FGFR1. Moreover, the relative number of FGFR1-containing epithelial cells was statistically significantly higher than in the patients with cleft palate (z=2.875; p=0.004). We found immunoreactive mucosal epithelial cells, cells of hair follicle, sweat and sebaceous glands. Relative amount of FGFR1 containing connective tissue cells was variable, but in the walls of blood vessels mainly was seen moderate number of immunoreactive structures. In general we observed statistically significant differences when compared the relative number of immunoreactive structures in cleft lip and cleft palate affected tissue (z=2.853; p=0.0043). Besides, we found plausibly weak correlation with bFGF (p=0.021; r=0.236).

DISCUSSION

TGFβ has regulatory effects on broad spectrum of cell types. It regulates cell replication and differentiation, angiogenesis and cellular migration (10). All of these processes are important not only in oral development, but also in wound healing. Palatal connective tissue is dense and basicly is composed of bundles of collagen fibers. TGFβ stimulates the synthesis of collagens (10). Thereby we can explain pronounced expression of mentioned growth factor exactly in cleft palate affected tissue. However, the dark side of the TGFβ effects is deposition of extracellular matrix in the sites of injury, what can lead to scarring and fibrosis (24). In general cleft palate affected tissue show more pronounced expression of

TGFβ than cleft lip disordered tissue and this difference is statistically significant (z=2.127; p=0.033). We can suppose that it depends from heaviness of malformation and such moderate expression of TGFβ in cleft palate tissue is evaluated as good tissue remodelling fact.

The type III receptor (TGFBR3 or betaglycan) was originally characterized as a coreceptor for TGFBR2. While TGFBR3 does not have functional kinase domain, it binds all three TGFβ isoforms and regulates their ability to interact and signal through other TGFβ superfamily signalling receptors (25). Immunohistochemical investigations of developing mouse palate in vivo and in vitro show that the expression of TGFBR3 was not identified in the palate during initial morphogenesis, but dramatically increased at the time of midline epithelial seam formation. So the expression of TGFBR3 was temporo-spatially restricted to a subgroup of palatal epithelial cells and correlated with the process of MEE epithelial-mesenchymal transformation (13). Expression of TGFBR3 in epithelium we didn't found in any control material, while in cleft palate disordered tissue it was seen in three cases together with expression in connective tissue and in the walls of blood vessels and was quite prominent. On the basis of our results we can speculate that exactly epitheliocytes of mentioned patients demonstrate immature phenotype, what is good signal for successful tissue remodelling after surgical operations. From other hand, in general cleft lip affected epithelium with slight, but significantly pronounced expression shows better transformation possibilities.

In the development of clefts, especially cleft palate, very important is also sufficient proliferation of mesenchymal cells and synthesis of extracellular matrix components. Thus we can hypothese that this is weak and insufficient palate connective tissue stimulating process because TGFBR3 is required to allow (by increasing the affinity) the binding of TGFBR2 to the various TGFβ isoforms (12).

A recent investigations show that TGFBR3 has an essential role in multiple human cancers. Thus scientist from China report, that the loss of TGFBR3 expression in human oral epithelium and stroma is common event in oral squamous cell carcinoma. Using immunohistochemistry and semiquantitative counting method they demonstrate moderate to intense expression in oral normal squamous epithelium (26). Mentioned findings confirm our results about week expression of TGFBR3 in cleft disordered tissue.

BMP – mediated mesenchymal proliferation is a key event in palatogenesis (17, 27). Suzuki with colleges analyze oral and maxillofacial area of human embryo immunohistochemically and conclude that similar in mouse and rat embryos BMP 2/4 is mainly localized in jaw bone, nasal epithelium, striated and smooth muscle (28). Another scientists report that immunostaining of BMP 2/4 is weak and not consistent in normal oral mucosa, but increase in the cases of oral carcinoma (29). Our results demonstrated statistically significant difference between relative amount of BMP 2/4 containing structures in cleft palate and cleft lip disordered tissue at all as well as separately in epithelium and connective tissue. We suppose that cleft palate affected tissue demonstrate more pronounced compensatory ability and epithelial cells facilitate cells of mesenchymal origin. It is very important that BMP 2/4 also have the ability to induce mesenchymal cells triggering their differentiation into osteoblasts (30).

Expression of bFGF in cleft disordered tissue in general was weak. It is known that expression of basic FGF in fibroblasts and endothelial cells increases in oral submucous fibrosis (31). Also during wound healing in skin the basal keratinocytes and basal cells of hair follicle notably express bFGF (32). Moreover, oral wounds heal significantly faster than skin and the number of bFGF positive cells in oral mucosa increase also faster than in skin (33). All mentioned results indicate significant role of mentioned growth factor in tissue remodeling during physiological and patological processes, especially in the oral mucosa. Cleft lip affected showed a little pronounced relative abundance of immunoreactive epithelial cells while tissue from cleft palate showed little pronounced relative abundance of positive connective tissue cells and exactly this difference was evaluated as statistically significant. Accordingly cleft affected tissue, especially cleft lip disordered tissue show deficiency of bFGF, however connective tissue from cleft palate demonstrate more possibilies of remodeling.

Cleft lip affected tissue demonstrated more statistically significantly prominent expression of FGFR1 in general as well as separately in epithelium. FGFR1 is the most important receptor in the FGF/FGFR system, it has the highest affinity for bFGF and it is widely distributed in the human body (34). Basic FGF/FGFR binding induces a broad spectrum of activities into the cells (35). Thus we can speculate that cleft tissue through expression of FGFR1 demonstrate conditioned readiness for remodelation, what is impossible because of slight expression of bFGF, except connective tissue from cleft palate. In general connective tissue from cleft palate affected tissue showed more equivalent

expression of mentioned growth factors and its receptors.

CONCLUSIONS

The marked and statistically significant expression of BMP 2/4 in cleft palate disordered soft tissue probably is delayed, but still proliferation and differentiation as well as tissue, especially, bone remodeling contributing signal, caused by insufficient palate hard tissue deficiency.

Cleft palate affected tissue show more prominent expression of TGFβ, still the weak regional expression of TGFβ type III receptors prove the disordered tissue growth and changed TGFβ signalling pathway in postnatal pathogenesis what can lead to formation of defective mucosa after surgical closure of malformation.

Expression of FGFR1 is most characteristic in cleft lip disordered tissue, however bFGF/FGFR1 system seems plays slight functional role only in cleft palate affected connective tissue what is probably insufficient in the case of palate defect.

In general, expression of TGFβ, BMP 2/4 and FGFR1 is significantly different, giving evidence to the involvement of this mentioned factors in the cleft severity morphopathogenesis.

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