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ORIGINAL ARTICLE

Expression of Gene Runx2, Wnt and OPG in Palate Cleft Reconstruction Material

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Summary
Introduction. Facial morphogenesis occurs from the fourth to the twelfth gestation week, when the cells from nerve crest migrate to the region of face, forming the primary palate. The cleft palate is an abnormality in embryogenesis period, which is characterized by the absence of fusion of palatal shelves. The incidence of cleft lip and palate is one in 700 live births. In recent years the effect of different genes and signaling molecules, including Runx2, Wnt3 and OPG have been studied in the development of cleft palate, because these substances are considered to be regulators of pathogenesis responsible for formation of bone and cartilage tissue and particularly the bone.
Aim of the Study. The aim of the work was to evaluate the expression of Runx2, Wnt3 and OPG in palate bone and nasal cartilage for children with cleft palate.
Material and methods. Eleven bone and cartilage samples were obtained from 21 children of the lip, soft and hard palate correction surgery. All the patients were diagnosed with clefts of the lip, alveolar process of maxilla, and palate. In the tissue sections using the immunohistochemistry method (IMH), were determined Runx2 (code: AB192256, 1: 250, Abcam GB, rabbit), Wnt3 (code: AB1992, 1: 800, Abcam GB, rabbit), and OPG (code: A0611, 1: 100, The Orbit USA, rabbit) local expression. We used a semi-quantitative census method for quantifying the positive structures.
Results. Runx2 expression was observed in five patient bone tissue samples and six patient cartilage tissue samples. Of the Runx2 positive bone tissue, in one case we observed occasional, in two cases- few, in one case- moderate to numerous and in one case numerous positive osteocytes while in tissue of cartilage in two cases we observed few, in one case- few to moderate, in two cases-moderate, and in one case numerous positive chondrocytes. A significant difference in Wnt3 expiation was observed between bone and cartilage tissues. Wnt3 expressing chondrocytes were observed in all samples, where in one case- occasional, in three cases-few, in one case-moderate, and in six cases-numerous positive cartilage cells were observed. The expression of the gene in the bone was observed in nine cases, which contained mostly occasional or few positive structures, except in three cases where in one Wnt3 was marked by few to moderate and in two cases numerous positive osteocytes. OPG expression was observed in all samples, but in the cartilage, the expression was more pronounced. In the cartilage in seven cases, there were numerous positive chondrocytes, in one case- few to moderate, in two cases moderate to numerous and in one case few to moderate number of chondrocytes. OPG showed variable expression. In four cases, we observed occasional to few, in one case few to moderate, in one case- moderate, in one case moderate to numerous and in four cases numerous positive bone cells.
Conclusion. Cartilage tissue expresses significantly more Runx2, Wnt3 genes and OPG proteins, indicating a greater compensatory tissue capacity. In the case of palate clefts, the high expression of Wnt3 and OPG and lower expression of Runx2 could indicate a significant tissue proliferation which predominates over mineralization and ossification processes.
Key words: cleft, cleft palate, osteocytes, chondrocytes, Runx2, Wnt3, OPG

INTRODUCTION

The cleft palate is an abnormality in the embryogenesis period, characterized by a loss of fusion of palatal shelves (1). The lip and palate clefts are found in one in 700 live births worldwide (17). About 70% of all lip and palate clefts are nonsyndromic, while the remaining 30% are associated with other hereditary abnormalities. The largest number of nonsyndromic lip and palate cleft is found in North American Indian and Asian populations, where incidence ranges from one to 500 in live birth children. The interaction of teratogenic factors and genes is important for the development of the lip and palate. In the case of clefts, the effects of different locuses have been shown in chromosomes 1, 2, 3, 4, 6, 18, 19 and 21, but the main teratogenic factors are maternal stress, alcohol use, smoking and the effect of folic acid in pregnancy (16).

In recent years, attention has been paid to specific genes and proteins in the process of cleft development, which directly affects the morphological processes of tissues (21).

Runx2 belongs to the Runx gene family, which is responsible for encoding transcription factors, mainly by engaging in cell differentiation (4). There are three distinct Runx genes. Runx1 is involved in hematopoiesis, Runx2 in sceletogenesis, but the role of Runx3 has not been fully understood (18). Runx2 primary importance is in osteoblast differentiation and cartilage hypertrophy in the primary palate, as well as Runx2 is associated with cell migration and bone vascularisation (3). The Runx2 gene in the bone mainly promotes mineralization and stimulates osteoblast proliferation (6). Researches have shown the effect of the osteoblast specific transcription factor Runx2

on the differentiation of mesenchymal stem cells to primary osteoblasts in cartilage tissue (22). Runx2 in immature chondrocytes promote cartilage hypertrophy (13). Runx2 are expressed mainly by hypertrophic perichondrocytes, which indicate the initial ossification process (8). Runx2 has also been shown to play a role in osteoarthritis, affecting mesenchymal cells. This gene promotes the activity of osteoblasts and chondrocytes, thereby promoting ossification processes (19).

Wnt3 is a determinant gene of signaling molecules that belongs to the Wnt gene family and regulates various morphogenetic processes, including cellular polarity detection, differentiation and migration (14). In the embryogenesis period, the role of Wnt3 is in bone mass formation through mesenchymal cell proliferation, regeneration of stem cells, and stimulation of preosteoblastic replication. In this developmental period, Wnt3 is believed to be involved in inhibition of osteoblast and osteocyte apoptosis and stimulation of developing the osteoblasts. The Wnt3 gene affects mesenchyme, causing it to form into osteoblasts (9). The Wnt3 gene in in cartilage tissue inhibits its proliferation and triggers the process of differentiation of the chondrocyte, which later, through involvement of other genes, will contribute to the ossification process (7). Recent studies have shown the direct activating effect of the Wnt3 gene signaling molecules on the active component of T cell transcription factor 1 (TCF1),which is a Runx2 gene promoter, thus activating the Runx2 gene in mesenchymal cells (5). In recent years, the Wnt3 gene is increasingly associated with lip and palate cleft, but the involvement of the gene in the pathogenesis mechanism is not completely clear (15).

Osteoprotegerin (OPG) is a protein belonging to the Tumor Necrosis Factor (TNF) gene family. Mostly, these proteins are synthesized by osteoblasts and play an important role in bone remodeling (11). OPG binds to the receptor activator of nucleic factor kappa B ligand (RANKL) to form the OPG / RANKL complex, prevents it from binding to the receptor activator of nuclear factor kappa B (RANK), which is present on the osteoclast cell surface, thereby inhibiting osteoclast differentiation and proliferation (20). OPG and RANKL play a primary role in bone metabolism process. Expression of OPG synthesis increases OPG / RANKL complex ratio, resulting in reduced bone remodeling (11). Tissue stress plays an important role in the inhibition of osteoclast and bone proliferation. Tissue stress is carried out through bone tissue metabolism, where OPG synthesis increases. In various studies, bone tissue stress is associated with tissue impairment, ultrasound, gravitation and mechanical damage. The increased OPG / RANKL ratio is associated with Paget's disease, benign and malignant bone tumors, metastatic processes, rheumatoid arthritis and postmenopausal osteoporosis (10). The effect of OPG on the development of palate clefts has been studied very little so far. OPG expression in palate bone tissues in children with lip and palate clefts has been demonstrated, suggesting osteoclast dysregulation in the morpho-pathogenetic process of the cleft (12). The WNT gene is believed to increase the level of OPG by reducing osteoclastogenesis and increasing the build-up of bone tissue in the cleft affected tissues (2).

AIM OF THE STUDY

The aim of the work was to evaluate the expression of Runx2, Wnt3 and OPG in palate bone and nasal cartilage for children with cleft palate.

MATERIAL AND METHODS

The material used in the study was obtained from lip, soft and hard palate correction surgeries in the Cleft Lip and Palate Centre of Institute of Stomatology of the Riga Stradiņš University from a period of time from 2003 to 2009. The study included 11 bone tissue and 11 cartilage tissue samples from 21 patients with clefts of the lip, alveolar process, hard and soft palate. The patient's age varied between seven and 21 years of age during the correction surgery.

The material obtained during the correction surgery was immediately fixated in transport test tubes with Stefanini (Zamboni) solution and delivered to RSU AAI Laboratory of Morphology for further tissue processing. After tissue fixation, the material was rinsed for 24 hours in a solution of Tyrode. The increase concentration of alcohol solution was used for tissue dewatering. The tissue was put into xylene for 30 minutes for degreasing. For the hardening the tissue was applied to the paraffin for 1 hour and in the paraffin for 2 hours.

Tissue blocks were cut into sections of 3 micrometers with semi-automatic rotary microtom (Leica RM2245, Leica Biosystems Richmond Inc., United States). Sections were fixed on slides and dried in a thermostat, later re-dewaxed in xylene and dehydrated in various alcoholic solutions. The tissue sections were dyed with hematoxylin and eosin to obtain a general overview. The immunohistochemistry method was used for the expression of genes and proteins in the tissues.

In the tissue sections, using the immunohistochemistry method, Runx2 (code: AB192256, working dilution 1: 250, Abcam, Great Britain, rabbit monoclonal), Wnt3 (code: AB1992, working dilution 1: 800, Abcam, Great Britain, rabbit polyclonal), and OPG (code: A0611, working dilution 1: 100, The Orbit, United States, rabbit polyclonal) local expression was obtained.

The semi-quantitative census method was used for the quantification of immunological structures (Pilmane 1997). The labels were as follows: (0) - no positive structure was detected in the visual field (0 / +) - occasional 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, (+++ / ++++) - numerous to abundance of positive structures seen in the visual field, (++++) – abundance of positive structures seen in the visual field.

RESULTS

The expression of Runx2, Wnt3, and OPG positive structures in the tissues was variable. Compared to bone tissue, the detection of genes and signal molecules was better observed in cartilage tissue, except in two cases where we observed moderate to numerous and numerous Runx2, Wnt3 and OPG positive cells in the bone tissue (see Table 1).

Runx2 expression was observed in five patient bone tissue and six cartilage tissue samples. From Runx2 positive bone tissue samples, in one case, we observed occasional, in two cases- few, in one case- moderate to numerous and in one case- numerous positive osteocytes (see Figure 1). On the other hand, in cartilage tissue, in two cases we observed occasional, in one case- few to moderate, in two cases- moderate, and in one case, numerous positive chondrocytes (see Figure 2).

A significant difference in Wnt3 expression was observed between the bones and cartilage. Wnt3 expressing chondrocytes were observed in all samples, in which one case we observed occasional, in three cases few, in one case- moderate and in six cases, numerous Wnt3 positive cartilage cells (see Figure 3 and Figure 6). The expression of the gene in the bone was observed in nine cases, which contained mostly occasional or few positive structures, except in three cases where Wnt3 was marked by few to moderate and in two cases- numerous positive osteocytes (see Figure 4).

OPG expression was observed in all samples, but expression in cartilage was more pronounced. In the cartilage in seven cases, there were numerous positive chondrocytes observed (see Figure 5), in one case – moderate to numerous, in two cases - moderate, and in one - few to moderate amount of chondrocytes. In the bones OPG was expressed variably. In four cases, we observed occasional to few, in one case – few to moderate, in one case- moderate to numerous, and in four cases, numerous positive bone cells.

DISCUSSION

In the bone, the Runx2 gene mainly promotes bone mineralization and osteoblast proliferation, thereby promoting bone modulation (6). The expression of this gene in cartilage shows an initial ossification (13). By promoting ossification, the development of the bone is also enhanced in the cleft palate. In our study, the expression of the Runx2 gene in bone was practically not observed, but the expression of gene in cartilage was weak, indicating a reduced mineralization of the tissues, but more active cartilage proliferation.

In our study, the presence of Wnt3 gene was observed in cartilage tissue, which indicates initial ossification in the cartilage. It has been shown that the Wnt3 gene mainly inhibits cartilage proliferation and induces chondrocyte differentiation (7), promoting bone development. Compared to the cartilage, the expression of Wnt3 in the bone was convincingly weaker, suggesting a weaker osteoblast involvement, thus weak bone mineralization. Studies have shown that the presence of the Wnt3 gene in osteoblast shows its proliferation (9).

OPG binds to the RANKL ligand to form the OPG / RANKL complex, in a result RANKL cannot bind to the RANK receptor located on the surface of the osteoclast cell, thereby inhibiting osteoclast differentiation and proliferation (20). The presence of OPG in the tissues indicates a reduction of cartilage and bone tissue reabsorption, promoting tissue development. In our study, we observed a significant presence of OPG, both in bone and in cartilage, indicative of compensatory tissue proliferation. We believe this is an essential early compensating mechanism in the case of cleft palate.

Since the expression of Wnt3 and OPG in cartilage tissue was more pronounced in comparison to bone tissue, except in two cases where in the bone all of these genes and proteins were observed in moderate and numerous osteocytes, therefore, we assume that tissue proliferation plays a more important role in the cleft palate, but tissue mineralization has less importance.

CONCLUSIONS

Increased cartilage tissue secretion of Runx2, Wnt3 genes and OPG proteins, proves probably the compensatory tissue capacity in case of clefts.

In the case of cleft lip and palate, the high expression of Wnt3 and OPG and less expressed Runx2 could indicate significant tissue proliferation predominance over mineralization and ossification processes.

Conflict of interest: None

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Table 1. The relative amount of Runx2, Wnt3 and OPG positive structures in the tissues obtained during the correction of lip, hard and soft palate

Nr.	Numeral	Runx2		Wnt3		OPG	
		Cartilage	Bone	Cartilage	Bone	Cartilage	Bone
1	48/1		0		0/+		+++
2	58/1		0		0		+
3	72/2		0		+/0		+
4	76/5		0/+		+		0/+
5	85 B/1		0		+		+/++
6	120		0		0		+
7	122/6		0		+		++
8	208/2		+		+/++		+++
9	239/2		+++		+++		+++
10	175/1		++/+++		+++		++/+++
11	15/3	0/+		0/+		+++	
12	15/5	0/+		+		+++	
13	22	+/++		+++		+++	
14	28	++		+		++/+++	
15	47/1	0		+		+++	
16	47/2	0	+	++	+	++	+++
17	58/2	0		+++		++	
18	58/4	++		+++		+++	
19	61/1	0		+++		+++	
20	64/1	0		+++		+/++	
21	110/7	+++		+++		+++	

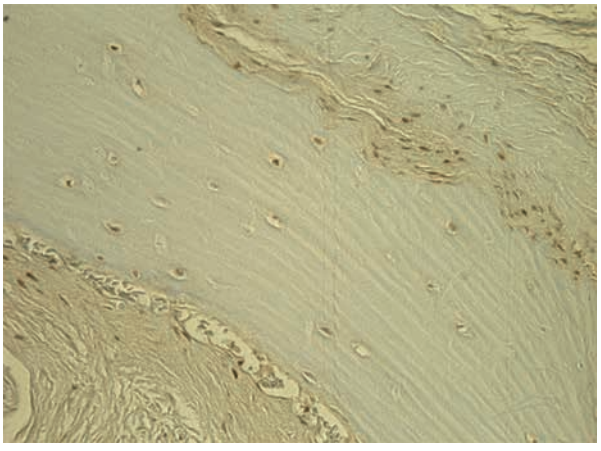

Fig. 1. Moderate to many Runx2 positive osteocytes in bone tissue for the patient with cleft lip and palate. Runx2 IMH x250

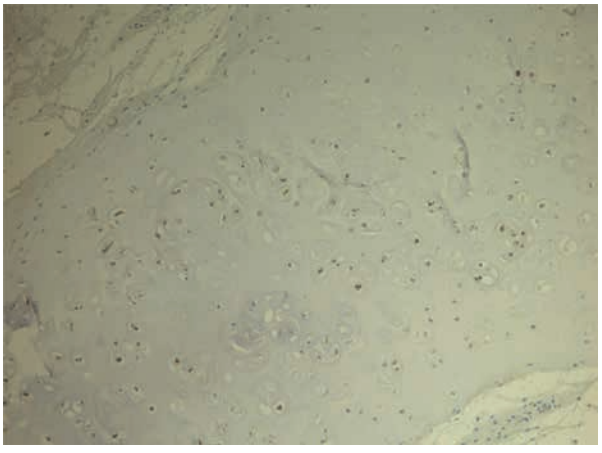

Fig. 2. Numerous Runx2 expressing chondrocytes in cartilage tissue for the patient with cleft lip and palate. Runx2 IMH x400.

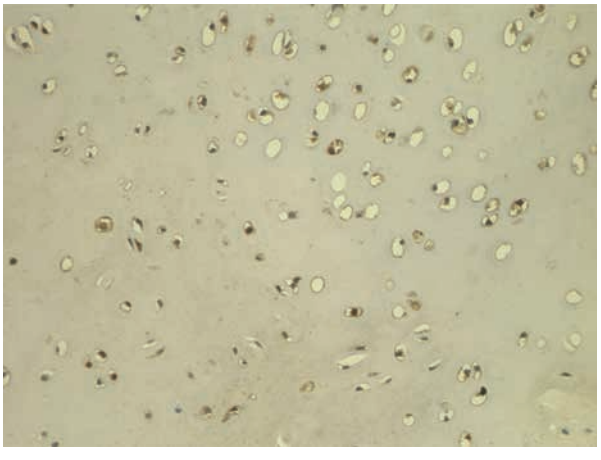

Fig. 3. Moderate to numerous Wbt3 positive chondrocytes in cartilage tissue for patient with cleft lip and palate. Wnt3 IMH, x250

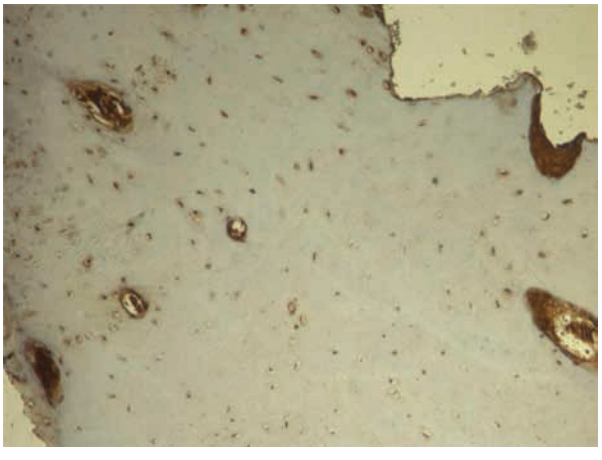

Fig. 4. Moderate to numerous Wnt3 positive osteocytes in bone tissue for patient with cleft lip and palate. Wnt3 IMH, x250

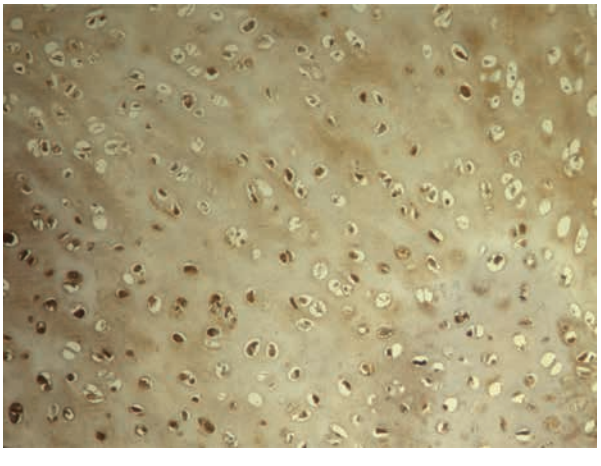

Fig. 5. Numerous positive chondrocytes in cartilage tissue for patient with cleft lip and palate. OPG IMH, x200

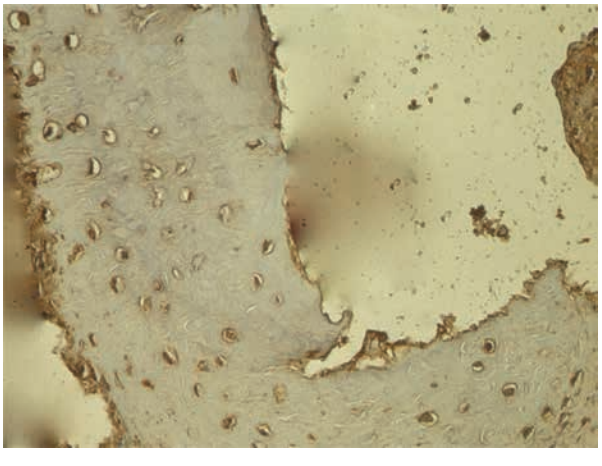

Fig. 6. Moderate to numerous Wnt3 positive osteocytes in bone tissue for patient with cleft lip and palate. Wnt3 IMH, x250