

Growth factors, apoptotic cells and *barx1* gene in bone and soft tissue of skeletal class III patients

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SUMMARY

Growth factors and growth stimulating genes are main signaling molecules for growth and development in ante- and postnatal period involved in cellular proliferation, differentiation and morphogenesis of tissues and organs during embryogenesis, postnatal growth and adulthood. The aim of this study was to evaluate TGF- β (transforming growth factor- β), BMP2/4 (bone morphogenetic protein 2/4), FGFR1 (fibroblast growth factor receptor one), *barx1* gene and apoptosis from tissue samples of oro-maxillo-facial region in skeletal class III patients to reveal possible morphopathogenesis of severe skeletal anomalies. The study group included 9 patients with skeletal class III malocclusion. During orthognathic surgery tissue samples from *tuber maxillae*, *ramus mandibulae* anterior and posterior part, as well as gingiva from the lower jaw in region of second molar have been taken. Samples were stained with immunohistochemistry for TGF- β , BMP2/4, FGFR1, apoptosis and *barx1* gene. We used also the routine histological staining with haematoxyline and eosine.

In *tuber maxillae*, *ramus mandibulae* anterior and posterior part staining for TGF- β was the most relevant. Also BMP2/4, FGFR1 and *barx1* showed the highest mean number of positive cells in *tuber maxillae*. *Barx1* was equally expressed in *ramus mandibulae*, but BMP2/4 and FGFR1 mainly stained its posterior part cells. Apoptosis mostly affected *ramus mandibulae* anterior part.

Conclusions. We suggest about more active stimulation of bone growth in *tuber maxilla* whereas *ramus mandibulae*. Apoptosis mainly affects *ramus mandibulae* anterior part that possibly connects to the lower expression of growth stimulating factors and may indicate lower bone remodeling ability.

Key words: growth factors, apoptosis, orthognathic surgery, immunohistochemistry.

INTRODUCTION

Patients with severe dentofacial anomalies are 5-7 % in Latvian population around the age 18 [1]. These anomalies cause functional as well as estheti-

cal disorders and decrease life quality for person. The etiopathogenesis of severe skeletal anomalies can be genetically determined and/or linked with postnatal development. The treatment of severe dentofacial anomalies is combined and related to environmental mechanism of bone development. It does consist of orthodontical procedures and orthognathic surgery, including mainly osteotomies with movement of divided jaw parts, fixation on right position, with following long-term consolidation and remodeling process. However, even on modern technologies based treatment can't exclude relapses after orthognathic treatment. Skeletal stability after orthognathic surgery depends on many factors and clinical data show that relapses differ individually without any clear seen origin. According to Mobarak et al. [2] relapse after jaw osteotomies is detected in around 10-30 % of cases. The main relapse occurs in the first 6 months after surgery. There may be mechanical and biological fac-

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tors substantial for relapse. Mechanical strain influences skeletal form via atrophic and hypertrophic mechanism that is deemed bone remodeling. To understand the differential morphology and physiology of craniofacial bones, it is essential to understand the fundamental intrinsic and extrinsic factors that guide growth, development and long-term maintenance. The genome codes for three fundamental mechanisms influencing bone morphology: growth factors, vascular induction and mechanically induced inflammation [3].

Essential role in bone remodeling play the growth factors [4]. Growth factors are main signaling molecules for growth and development in ante- and post-natal period. They are involved in cellular proliferation, differentiation and morphogenesis of tissues and organs during embryogenesis, postnatal growth and adulthood [5]. The effect of growth factors is mediated through surface receptors (juxtacrine effects) on the target cells by activating of intracellular phosphorylating enzymes that induce an intracellular signaling pathway by aggregation of co-factors and other to nuclei migrating proteins. Together with other transcription factors they activate a set of genes, which then exert the specific changes in cellular activity or phenotype [6].

Transforming growth factor- β (TGF- β) belongs to a large superfamily of related proteins that also includes bone morphogenetic proteins (BMP), growth and differentiation factors, activins and inhibins. During the early stages of bone formation, the action of TGF- β is to recruit and stimulate osteoprogenitor cells to proliferate, providing a pool of early osteoblasts [7]. Quite the opposite, during later phases of osteoblast differentiation, TGF- β blocks differentiation and mineralization [8].

Marchal Urist in the 1960's was the first who reported that protein extracted from demineralized bone matrix were able to induce bone formation at ectopic sites in rodents and the process initiated by the implantation of these extracts closely resembled the cellular progression seen during endochondral bone formation and fracture healing [9]. Urist called this bone-forming activity bone morphogenetic protein [10]. BMP passes the unique functions of inducing the differentiation of cells in the osteoblastic lineage, therefore is increasing the pool of mature cells and enhances the differentiation function of the osteoblast [11; 12; 13; 14].

FGF (fibroblast growth factor) and FGFR (fibroblast growth factor receptors) play a critical role in morphogenesis by regulating of cell proliferation, differentiation and cell migration during embryonic development. In adult organism FGF play an important role in the control of the nervous system, in tissue repair and wound healing [15].

Interesting, that influence of growth factors is also regulated by different events, from which one of most important is apoptosis [16; 4].

Expression of *barx1* gene was observed in craniofacial mesenchyme and could be associated with skeletal hypoplasia in dentofacial region [17].

The aim of this article is present a progress report of evaluation of TGF- β (transforming growth factor- β), BMP2/4 (bone morphogenetic protein 2/4), FGFR1 (fibroblast growth factor receptor one), *barx1* gene and apoptosis from tissue samples of oromaxillo-facial region in skeletal class III patients to reveal possible morphopathogenesis of severe skeletal anomalies.

MATERIALS AND METHODS

Preliminary study group included 9 patients (5 females and 4 males) with severe skeletal class III anomalies who needed combined orthodontic treatment and orthognatic surgery. The average age of patients was 19.6 years. During orthognatic surgery tissue samples were obtained from *tuber maxillae*, *ramus mandibulae* anterior and posterior part and gingiva from the lower jaw in region of second molar. The samples were fixed in 2% formaldehyde and 0.2% picric acid 0.1 M phosphate-buffer (pH 7.2). After they were washed in phosphate-buffered saline for 12 hours, embedded in paraffin, and cut into sections of 6-7 μ m thickness. After deparaffinization slides were stained with biotin-streptavidin immunohistochemical (IMH) method [18] for transforming growth factor- β (TGF- β , 1:100, *RD Systems*, UK), bone morphogenetic protein 2/4 (BMP2/4, 1:100, *RD Systems*, UK), fibroblast growth factor receptor 1 (FGFR1, 1:100, *Cambridge Science Park*, UK) and *barx1* gene (1:250, *Cambridge Science Park*, UK).

TUNEL kit (*Roche Diagnostics*, Germany) was used to detect apoptotic cells [19]. To get the overall review we used the routine histological staining with haematoxyline and eosine.

Counting of positive cells per 4 visual fields and calculation of mean value and standard deviation performed semi-quantative analysis of the slides. The statistical evaluation of the findings was performed with the help of SPSS for Windows 10.0 software. The study protocol was approved by the ethics committee of Riga Stradins University.

RESULTS

In bone from *ramus mandibulae* anterior and posterior part we detected osteones of different size,

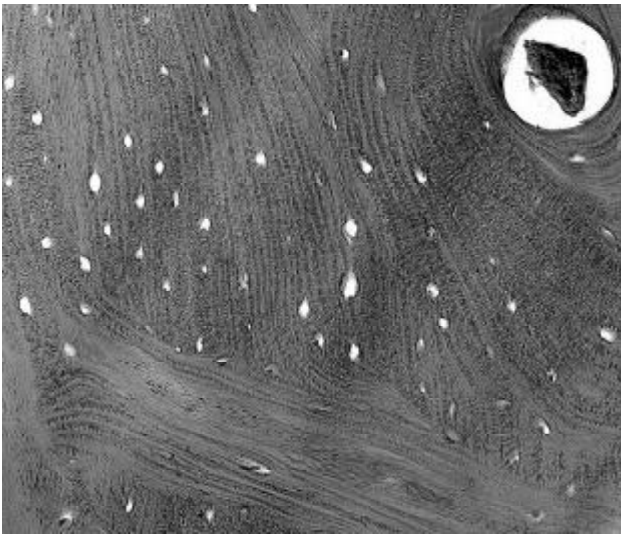


Fig. 1. Microphotograph of bone from ramus mandibulae anterior part (haematoxylin and eosine; x 250). Irregular distribution of osteone structures

irregularly orientated cells and bone lamellae (Fig. 1). However, the bone from *tuber maxillae* seemed even more chaotically organized with some circular lamellar structures only in osteones (Fig. 2). Some regions showed hyperplasia of connective tissue in the Haversian channels. Blood vessels in soft tissue close to such bone fragments and in *periosteum* were with signs of sclerotization.

Osteocytes of *ramus mandibulae* anterior and posterior part intensively expressed BMP 2/4, although in posterior part of bone positive cells showed more regional distribution than in anterior one (Figures 3, 4). Gingival epithelium above the affected bone regions demonstrated hyperplasia of basal cells and vacuolization of polymorphous epitheliocytes in superficial layers (Fig. 5). Basal epithelial cells also ex-

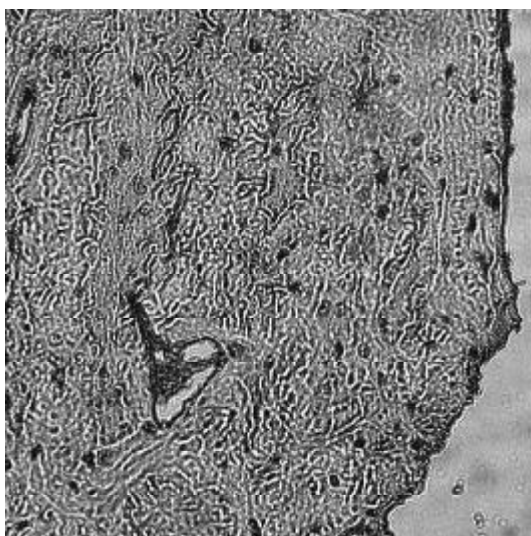


Fig. 3. Microphotograph of bone from ramus mandibulae anterior part (immunohistochemical examination; x 250). Expression of BMP2/4

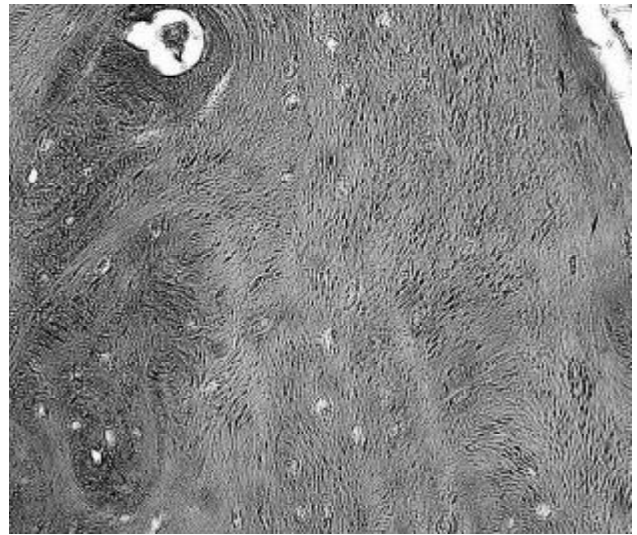


Fig. 2. Microphotograph of bone from tuber maxillae (haematoxylin and eosine; x 250). Chaotically organized bone structure

pressed *barx1* (Fig. 6). *Tuber maxillae* osteocytes expressed FGFR1 (Fig. 7) and also intensive expression of *barx1* was detected in osteogenic and periosteal cells (Fig. 8). Regional distribution of *barx1* was seen also in *ramus mandibulae* anterior and posterior parts (Fig. 9).

In tissue sample from *tuber maxillae*, *ramus mandibulae* anterior and posterior part staining for TGF- β was the most evident (122.25 ± 8.220 cells/mm², 93 ± 11.401 cells/mm² and 69.5 ± 12.503 cells/mm², respectively; see Table; Fig. 11). Also BMP2/4 showed high mean number of positive cells in *tuber maxillae* (65.5 ± 17.233 cells/mm²) and *ramus mandibulae* posterior part (43.25 ± 6.625 cells/mm²), but less positive cells expression was seen in *ramus mandibulae* anterior part (26 ± 6.164 cells/mm²), see Fig. 12.

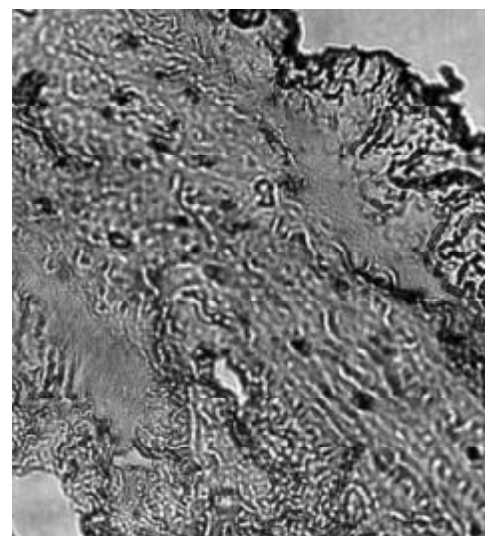


Fig. 4. Microphotograph of bone from ramus mandibulae posterior part (immunohistochemical examination; x 250). Expression of BMP2/4

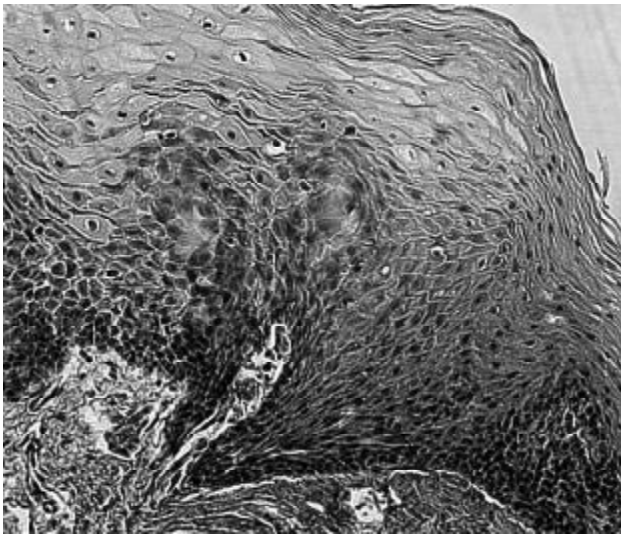


Fig. 5. Microphotograph of oral mucosa from region of lower second molar (haematoxylin and eosine; x 400). Hyperplasia in basal epithelial cells and vacuolization in more superficial layer

More FGFR1 expression also was observed in tissue sample from *tuber maxillae* (44.25 ± 15.217 cells/mm²) and *ramus mandibulae* posterior part (28.25 ± 13.301 cells/mm²) compared with *ramus mandibulae* anterior part (18.25 ± 3.593 cells/mm²), see Fig. 13. In skeletal class III patient *ramus*



Fig. 6. Microphotograph of oral mucosa from region of lower second molar (immunohistochemical examination; x 250). Expression of barx1 gene

mandibulae anterior part was noticed the large amount of apoptotic cells (44.75 ± 8.52 cells/mm²) that decreases in *tuber maxillae* and *ramus mandibulae* posterior part (28.25 ± 3.403 and 21 ± 9.933 cells/mm²; Fig. 10). *Barx1* gene expression was highest in tissue sample from *tuber maxillae* (22.75 ± 6.5 cells/

Table. Mean numbers and standard deviation (SD) of TGF- β , BMP2/4, FGFR-1, TUNEL and barx1 positive cells in bone of orthognatic surgery skeletal class III patients

Place	Factors	TGF - β	BMP2/4	FGFR-1	TUNEL	barx 1
<i>Tuber maxilla</i>		122.25 \pm 8.220	65.5 \pm 17.233	44.25 \pm 15.217	28.25 \pm 3.403	22.75 \pm 6.5
<i>Ramus mandibulae anterior part</i>		93 \pm 11.401	26 \pm 6.164	18.25 \pm 3.593	44.75 \pm 5.852	6 \pm 1.154
<i>Ramus mandibulae posterior part</i>		69.5 \pm 12.503	43.25 \pm 6.625	28.25 \pm 13.301	21 \pm 9.933	6.75 \pm 4.856

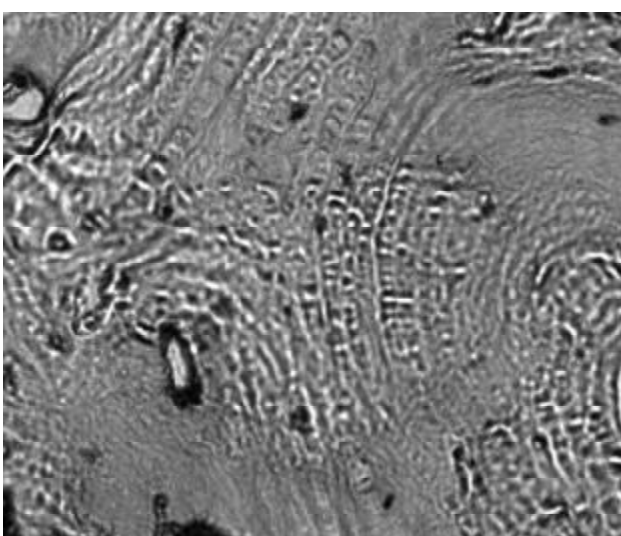


Fig. 7. Microphotograph of bone from tuber maxillae (immunohistochemical examination; x 250). Expression of FGFR1

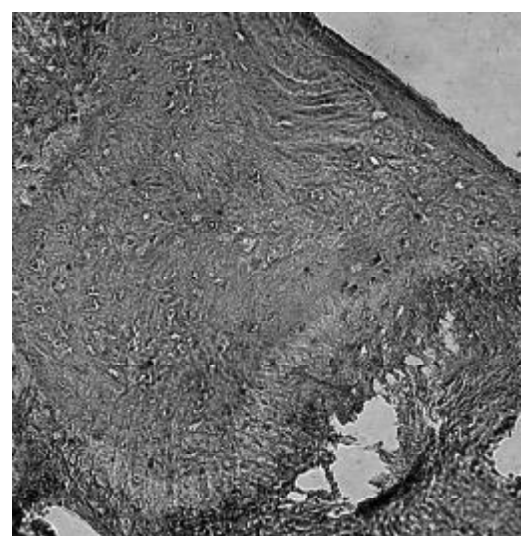


Fig. 8. Microphotograph of bone from tuber maxillae (immunohistochemical examination; x 250). Expression of barx1 gene

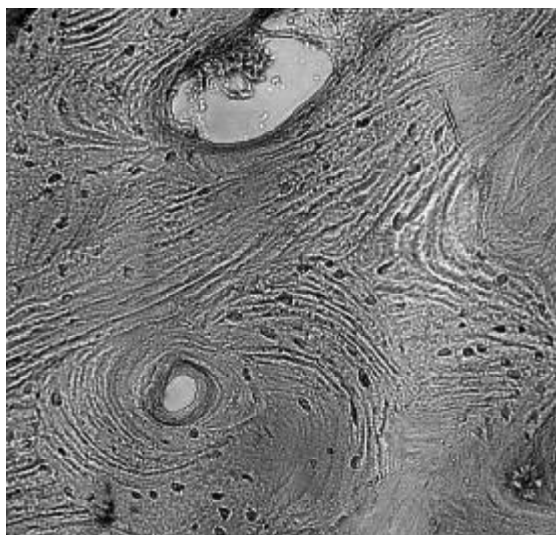


Fig. 9. Microphotograph of bone from ramus mandibulae anterior part (immunohistochemical examination; x 250). Expression of barx1 gene

mm²) while *ramus mandibulae* anterior and posterior part showed almost equal mean numbers of positive cells (6 ± 1.154 and 6.75 ± 4.856 cells/mm²), see Fig. 14.

There were statistically significant differences between mean numbers of growth factors, *barx1* containing and apoptotic cells in *tuber maxillae*, *ramus mandibulae* anterior and posterior part.

DISCUSSION

Preliminary results from this study suggest that bones from various sites in maxilla and mandible show different expression of growth factors, *barx1* gene and apoptotic cells. There is almost no data about TGF- β , BMP2/4, FGFR1, *barx1* and apoptotic cells

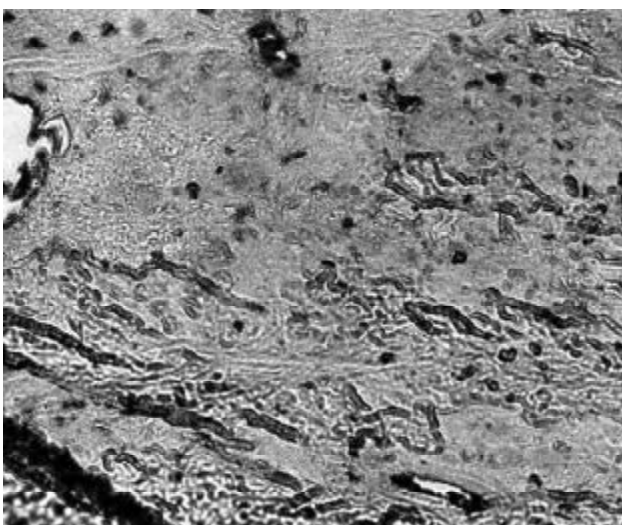


Fig. 11. Microphotograph of bone from ramus mandibulae anterior part (immunohistochemical examination; x 400). Expression of TGF- β

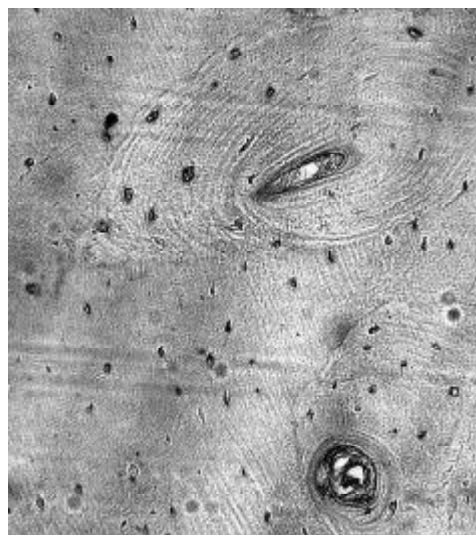


Fig. 10. Microphotograph of bone from ramus mandibulae posterior part (TUNEL method; x 250). Expression of apoptotic cells

distribution in orthognathic surgery patients in the scientific literature.

In all tissue samples TGF- β was seen to be most evident, possibly because this protein belongs to TGF- β superfamily that includes TGF- β themselves, activins and BMP. BMP 2/4 showed high level in tissue samples from *ramus mandibulae* anterior and posterior part as well as in *tuber maxillae* that partially support the data of Suttapreyasri et al. [4]. However most part of studies have reported just appearance of BMP in distraction osteogenesis and thus concluded that BMP enhance bone regeneration and remodeling [20; 21; 22; 23; 24].

FGFR1 was seen in smaller amount when compared with BMP2/4 in *tuber maxillae* and *ramus*

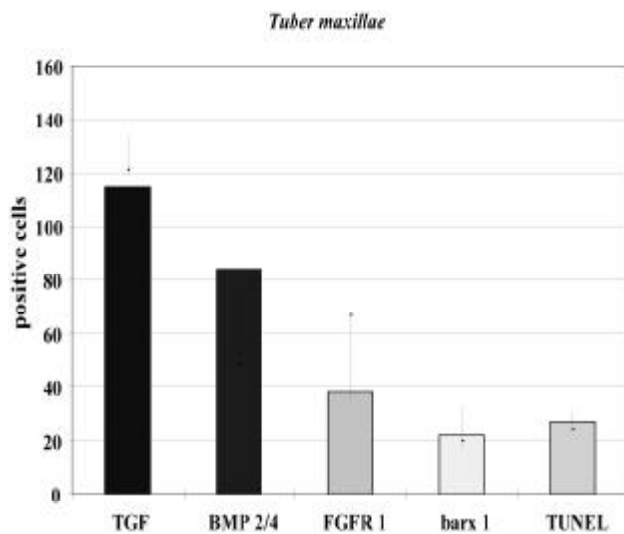


Fig. 12. Comparison of mean numbers in TGF- β , BMP 2/4, FGFR 1, barx 1 and apoptotic cells (TUNEL) in tissue samples from tuber maxillae

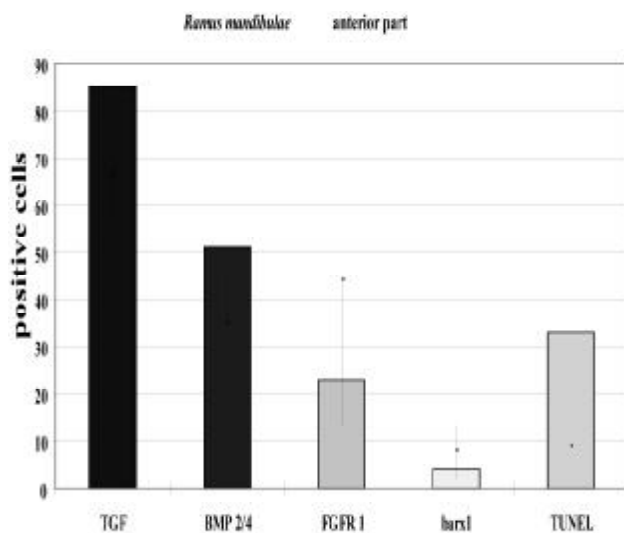


Fig. 13. Comparison of mean numbers in TGF- β , BMP 2/4, FGFR 1, barx1 and apoptotic cells (TUNEL) in tissue samples from ramus mandibulae anterior part

mandibulae posterior part, but almost equally in samples from *ramus mandibulae* anterior part of our patients. This growth factor is highly expressed in bone and cartilage of adult patients and plays an important role in hard tissue repair and regeneration process [15].

We used TUNEL method to detect apoptotic cells and this method is very effective at identifying nuclear DNA fragmentation associated with apoptosis [19]. In *ramus mandibulae* anterior part apoptotic cells were more observed than such expressing BMP2/4 and FGFR1 in the same tissues sample. That's mean that resorbition of bone in this region may be more active than new bone formation.

Indistinct expression of *barx1* [17] in all jaw sites seems to correlate with more distinct postnatal supportive tissue growth processes in jaw in cases of already appeared dentofacial anomalies, that does not need more mesenchyme stimulation.

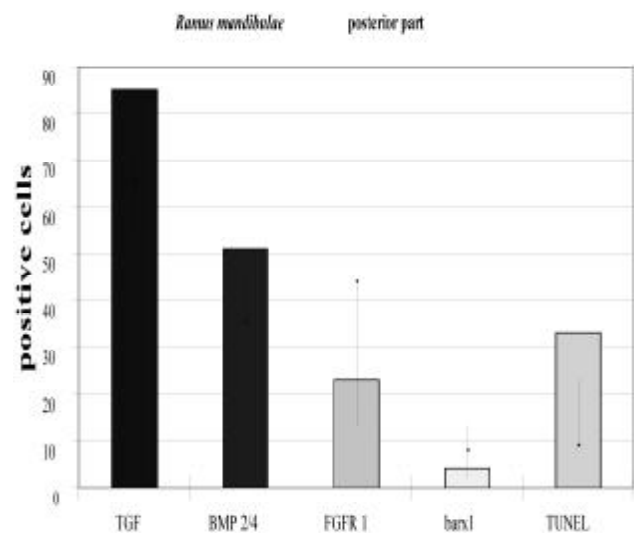


Fig. 14. Comparison of mean numbers in TGF- β , BMP 2/4, FGFR 1, barx1 and apoptotic cells (TUNEL) in tissue samples from ramus mandibulae posterior part

Concerning to our study it is envisaged that the results overall would lead to a better understanding of bone morphogenesis and remodeling potential at cellular, molecular and genetic level, so that clinically effective treatment strategies could be developed in future for patients with dentofacial anomalies and deformities.

CONCLUSIONS

In skeletal class III anomalies expression of growth factors and bone growth stimulating genes the highest is in *tuber maxillae*. Growing centers of *ramus mandibulae* possess equal *barx1* expression, while FGFR1 and BMP2/4 mainly stimulate *ramus mandibulae* posterior part, and TGF- β – its anterior part. Apoptosis mainly affects *ramus mandibulae* anterior part that possibly connects to the lower expression of growth stimulating factors and may indicate lower bone remodeling ability.

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