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ORIGINAL PAPER

The Role of RANK/RANKL/OPG Signalling Pathways in Osteoclastogenesis in Odontogenic Keratocysts, Radicular Cysts, and Ameloblastomas

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Abstract The aim of this study was to evaluate the immunohistochemical expression of molecules involved in osteoclastogenesis, including the receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) in odontogenic keratocysts (OKCs), which has been named as a keratocystic odontogenic tumour by the WHO, and compare their expression with radicular cysts and ameloblastomas. RANK is a member of tumour necrosis factor receptor family and it is activated by RANK ligand. OPG binds to RANKL and inactivates it. The imbalance of these factors could cause the differential bone resorption activity in some diseases and tumours. The expression of these molecules was evaluated in ameloblastomas ($n = 20$), OKCs ($n = 20$), and radicular cysts ($n = 20$) by immunohistochemistry. Immunohistochemical reactivity for RANK, RANKL, and OPG was detected in neoplastic and nonneoplastic epithelium and connective tissue cells. RANK showed the greatest expression in OKCs followed by ameloblastomas, with the lowest expression seen in radicular cysts. Expression of RANKL was detected in all lesions and no significant differences were observed between groups. OPG was expressed very low in all groups. In the stroma, the number of RANK positive cells was higher in OKCs when compared with ameloblastomas and radicular cysts but radicular cyst had higher numbers of RANKL positive cells in the stroma than ameloblastomas. The molecular system of RANK/RANKL/OPG is variably expressed in OKCs, radicular cysts, and ameloblastomas and this system may be involved in the osteoclastogenic mechanisms in OKCs and ameloblastomas. Advanced studies could further clarify the role of RANK, RANKL, and OPG in mediating tumour associated bone osteolysis.

Keywords Odontogenic keratocyst · Ameloblastomas · RANK · RANKL · OPG

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Introduction

The discovery of the role of receptor activator nuclear factor kappa B (RANK), receptor activator nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) molecular system in osteoclastogenesis has provided major advances in bone biology [1]. RANK, a member of the tumour necrosis factor receptor superfamily (TNFRSF), is a central activator of NF-kB, which controls the transcription of DNA, and is the signalling receptor for RANKL. RANKL is a homotrimetric protein and is typically membrane-bound on osteoblastic and activated T cells. RANKL binds to RANK on the surface of preosteoclasts and stimulates the development and activation of osteoclasts. OPG, also known as tumour necrosis factor receptor superfamily member 11B (TNFRSF11B), is secreted by many cell types, in addition to osteoblasts, including heart, kidney, liver, and spleen [2, 3]. OPG is a soluble decoy receptor for RANKL that inhibits the pro-osteoclastogenic interaction between RANKL and RANK, thereby inhibiting bone resorption [2–4]. Hence, its alternative name as osteoclastogenesis inhibitory factor (OCIF).

Therefore, the balance between OPG and RANKL regulates bone resorption and formation [5–7]. Imbalance of

the RANKL/OPG system have been implicated in the pathogenesis of various primary and secondary bone malignancies [8]. Moreover, they may be involved in regulation of the immune system, arterial calcification, and a number of metabolic bone diseases [1]. Some studies have also examined the roles of osteoclast regulatory factors in progression of odontogenic lesions [9–12].

OKCs are known for their extensive local invasion into adjacent structures and high recurrence rate after treatment [13, 14]. These clinical features are also seen in ameloblastoma, which show involvement of adjacent soft tissues and destructive growth. In addition to locally invasive behavior, both are characterized by a high rate of recurrence. For these reasons, OKC has been named "keratocystic odontogenic tumour" by the World Health Organization (WHO) Classification of Head and Neck Tumours in 2005 [15]. However, there is still no concensus about whether OKC is a benign neoplasm or not.

Radicular cysts are the most common inflammatory cysts of the jaws and arise from the proliferation of small odontogenic epithelial residues. They tend to be less than 1.5 cm in diameter and do not recur after appropriate management [13, 14]. In this study, we investigated the potential and mechanism of OKCs to induce osteoclastogenesis compared with ameloblastomas and radicular cysts.

Materal and Methods

Sample Selection

A total of 60 cases were collected from the tissue block archive of the Department of Tumour Pathology, at the Institute of Oncology, Istanbul University. Cases of OKCs, radicular cyst, and ameloblastoma diagnosed between January 2005 and January 2008, were reviewed by an oral pathologist (M.S.T.) and the most diagnostic cases were selected. These consisted of 20 parakeratotic OKCs (9 women, 11 men; mean age 46.5), 20 radicular cysts (8 women, 12 men; mean age 29.05), and 20 ameloblastomas (10 women, 10 men; mean age 31.2), including 11 follicular and 9 plexiform types. OKC cases associated with Nevoid basal cell carcinoma syndrome (NBCCS), orthokeratotic, and recurrent OKCs were excluded from the study. Similarly,recurrent ameloblastomas were excluded from the study.

All OKCs occurred in the mandible, with the posterior region and ramus the most frequent anatomic site, with 15 cases (75%), followed by the premolar region, with 4 cases (20%). Most ameloblastomas were located in the mandible (95%), including 15 cases (75%) in the posterior mandible and ramus and only one case arising in the maxillary molar region (5%). The majority of radicular cysts occurred in the maxilla (14 cases; 70%), with the anterior maxilla as the most frequent site, including 9 cases (45%).

Immunohistochemistry

For immunohistochemical studies, the paraffin blocks were cut serially into approximately 5 μm thick sections on charged slides. Firstly, the sections were penetrated and dried overnight in an autoclave (56°C). They were deparaffinized with xylene for 30 min, and washed with 99% alcohol for 15 min, then 96% alcohol and distilled water. Histostain-Plus Bulk Kit (Zymed 2nd Generation, LAB-SA Detection System, 85-9043, Carlsbad, CA, USA) was used for RANK and OPG, and Goat ImmunoCruz Staining System (Santa Cruz Biotechnology, sc-2053, Santa Cruz, CA, USA) was used for RANKL in this study. For antigen retrieval, the sections were microwaved four times for 5 min in citrate buffer (pH 6.0), cooled to room temparature, and then washed in phosphate buffered saline (PBS) for 5 min. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 and washed in distilled water, then soaked in PBS for 5 min. To prevent nonspecific reactions, sections were incubated with block solution. Nuclear factor kappa B (RANK) antibody at a dilution of 1:50 (Santa Cruz Biotechnology, H-300, Rabit, Monoclonal, sc-9072, Santa Cruz, CA, USA), nuclear factor kappa B ligand (RANKL) at a dilution of 1:50 (Santa Cruz Biotechnology, N-19, Goat, Polyclonal, sc-7628, Santa Cruz, CA, USA), and osteoprotegerin (OPG) at a dilution of 1:50 (Santa Cruz Biotechnology, CC912, Mouse, Monoclonal, sc-80259, Santa Cruz, CA, USA) were used as primary antibodies. Slides were incubated 120 min with RANK and OPG, and overnight with RANKL. Negative control sections treated with phosphate-buffered antibodies were confirmed to be unstained. The secondary antibody was reacted for 25 min., followed by streptavidin peroxidase reagent for 25 min. AEC (Zymed Laboratories, 00-2007, Lot No: 319293A, Carlsbad, CA, USA) chromogen was used to visualize the reaction. Finally, the sections were counterstained with Mayer's hematoxylin, coverslipped, and evaluated by a light microscope.

Evaluation Methods

The specimens were examined at 20× magnification with an Olympus BX60 microscope attached to a color video camera (Olympus Analysis Five) which connected to a computer. Images were captured using the camera and displayed on a computer monitor. The number of cells was evaluated in epithelium and connective tissue by an oral pathologist (M.S.T.). Moderate to high staining in the cell membrane and cytoplasm was interpreted as a positive cell.

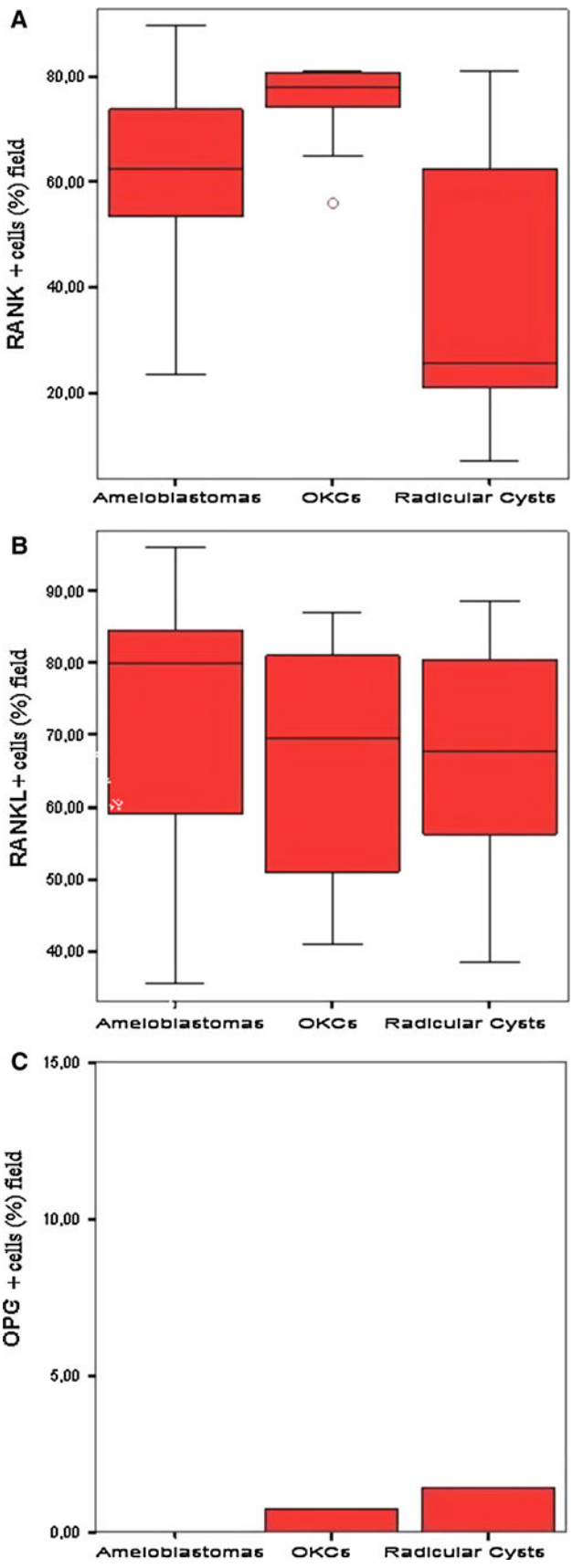


Fig. 1 Graphs of immunohistochemical staining for **a** RANK, **b** RANKL, and **c** OPG in odontogenic epithelium of ameloblastomas, OKCs, and radicular cysts. The *horizontal bar* within the *red zone* represents the median value

In the epithelium, positive cells were counted in 5 contiguous and consecutive microscopic high-power fields. In the stroma, positive endothelial, round, and fusiform cells (fibroblasts) were counted. Each field had an area of 0.0061 mm^2. All calculations were performed by the SPSS 16.0 (Statistical Package for Social Science). The statistical significance of differences in RANK, RANKL, and OPG reactivity were analyzed by the Mann–Whitney *U* test and *P* values <0.05 were considered to indicate statistical significance. Results were received as the mean of the percentage of positive cells determined for each field.

Results

Immunohistochemical Findings in the Odontogenic Epithelium of OKCs, Radicular Cysts, and Ameloblastomas

The results of immunophenotypic expression for RANK, RANKL, and OPG detection in odontogenic epithelium are shown Fig. 1a–c. Immunoreactivity for RANK, RANKL, and OPG was observed in the cytoplasm and plasma membrane of odontogenic epithelial cells in both neoplastic and nonneoplastic odontogenic lesions. No differences were detected between the basal layer and suprabasal layer of OKCs, radicular cysts, or in the inner and outer layers of tumour islands of ameloblastomas. RANK expression in the epithelial lining OKCs was significantly higher than in both the epithelial tumour islands of ameloblastomas and the epithelial lining of radicular cysts ($P < 0.01$, $P = 0.003$, respectively; Fig. 2). Ameloblastomas, in turn, had a higher number of RANK-positive cells than radicular cysts ($P = 0.003$).

Expression of RANKL was detected in all lesions and there were no statistically significant differences in the epithelial components of OKCs, ameloblastomas, and radicular cysts. All groups showed strong reactivity with RANKL (Fig. 3), whereas they showed almost complete lack of expression of OPG (Fig. 4).

Immunohistochemical Findings in the Connective Tissue Cells of OKCs, Radicular Cysts and Ameloblastomas

Expression of RANK, RANKL, and OPG in the connective tissue cells of the groups was also observed. The results are shown in Fig. 5a–c. Considering only immunoreactivity in

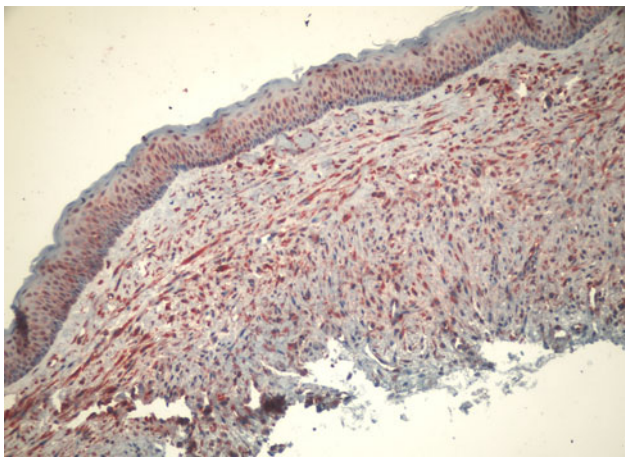


Fig. 2 OKC demonstrating RANK-positive cells in lining epithelial cells and connective tissue (original magnification, ×20)

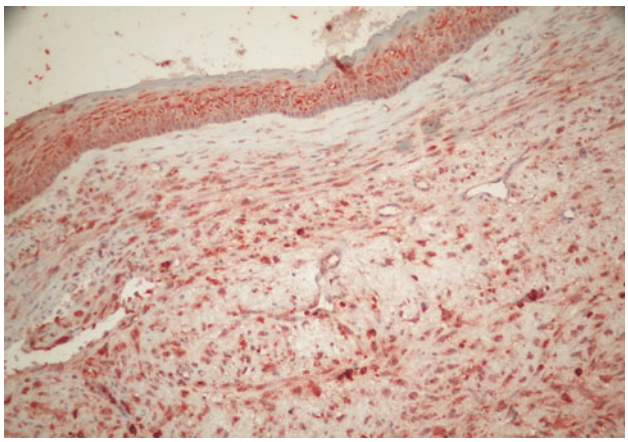


Fig. 3 Representative immunostaining for RANKL. OKC showed strong cytoplasmic and membranous staining in both connective tissue and odontogenic epithelium cells (original magnification, ×20)

the connective tissue cells of these lesions, OKCs exhibited higher number of RANK-positive cells when compared with ameloblastomas and radicular cysts ($P = 0.018$, $P = 0.003$, respectively; Fig. 2). Moreover, there was no statistically significant difference in the stromal reaction for RANK between ameloblastomas and radicular cysts.

All groups showed strong reactivity for RANKL. There were no significant differences in the expression of RANKL between connective tissue cells of OKCs and ameloblastomas or between OKCs and radicular cysts, whereas radicular cysts had higher numbers of RANKL positive cells than ameloblastomas ($P = 0.006$; Fig. 3).

Although OPG was expressed more strongly in ameloblastomas and radicular cysts than in OKCs ($P = 0.043$, $P = 0.030$, respectively), all groups showed very low expression (Fig. 4). Moreover, there were no statistically significant difference in the number of OPG-positive

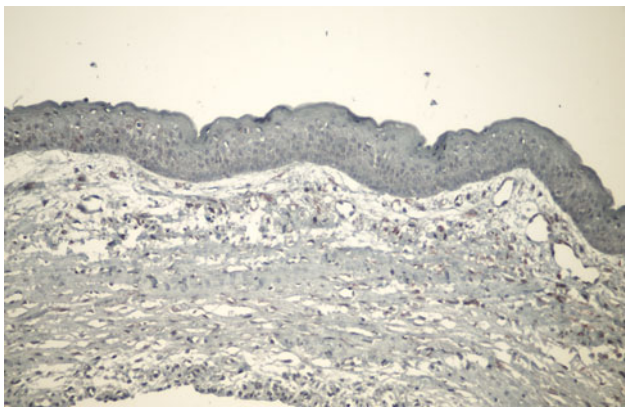


Fig. 4 Representative immunohistochemical staining for OPG. Almost no OPG staining was seen in the lining epithelium and connective tissue of this OKC. A similar low expression was seen in ameloblastomas and radicular cysts (original magnification, ×20)

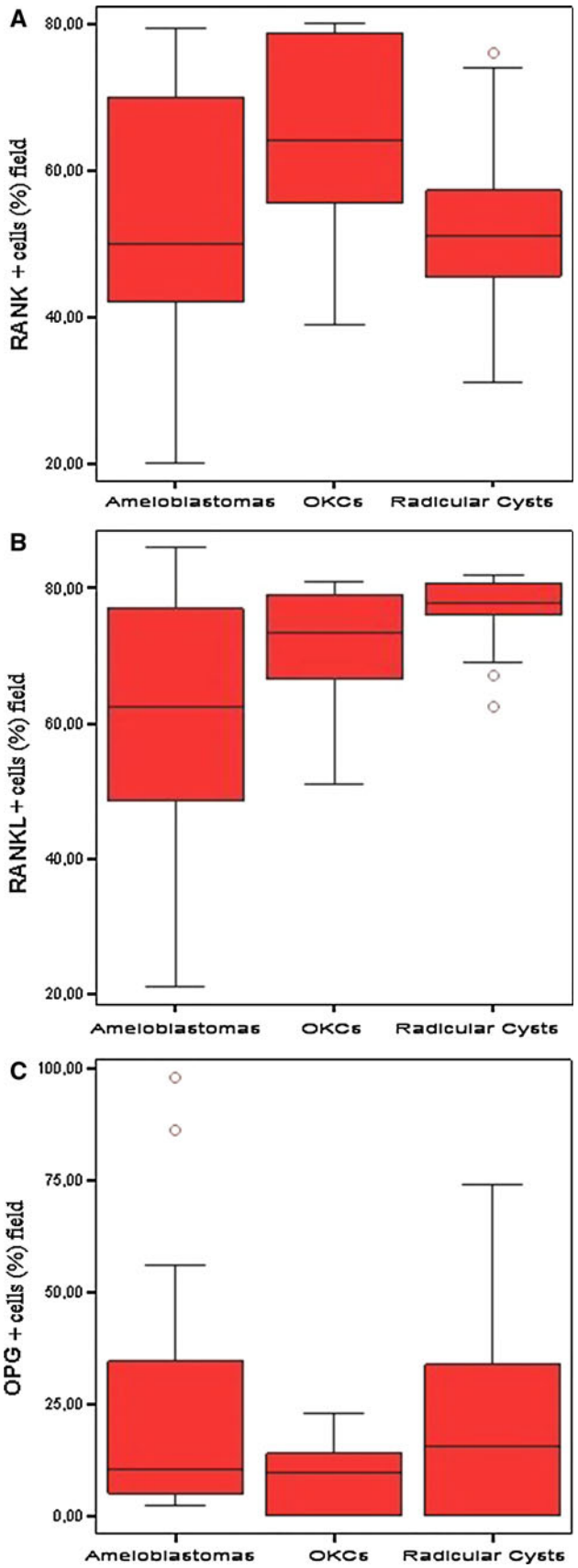
connective tissue cells of radicular cysts versus ameloblastomas.

Discussion

Much bone research is directed to better understanding the interaction of RANK, RANKL, and OPG, since the identification of these mediators in the signalling pathway of osteoclastogenesis. Bone resorption is a complex process initiated by the proliferation of immature osteoclasts, whose differentiation is principally regulated by the RANK/RANKL/OPG system. The interaction between RANK and RANKL plays a critical role in promoting osteoclast differentiation and activation, thus leading to bone resorption. OPG is a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting RANKL binding to RANK [16, 17]. OPG and RANKL have also been detected in odontoblasts, ameloblasts, pulp cell lines, and periodontal ligament cells and their expression is considered to play a role in osteoclastogenesis and bone resorption in the formation of a tooth eruption pathways [18–20].

Some of these recent studies are concerned with designing effective and rational drugs to treat bone-related diseases [21–23]. After all, osteoclastogenesis/bone resorption in periodontal disease can be ameliorated by inhibition of RANKL activity or by diminishing immune cell stimulation. Therefore, it is suggested that these two procedures, if localized, have the potential to lead to the prevention or therapeutic management of periodontal disease [24].

More recent studies have shown expression of RANK, RANKL, and OPG in the lining epithelium and walls of odontogenic cysts, including periapical cysts and



◀ **Fig. 5** Graphs of expression of **a** RANK, **b** RANKL, and **c** OPG in connective tissue cells of ameloblastomas, OKCs, and radicular cysts. The *horizontal bar* within the *red zone* represents the median value

granulomas [25]. Da Silva et al. [9] evaluated these bone resorption regulators in odontogenic lesions. They found that the stroma of OKCs exhibited a higher number of OPG-positive cells than RANKL-positive cells, whereas the opposite was observed in our study. The solid/multicystic type of ameloblastomas also had a greater number of RANKL-positive cells in the stroma than did OKCs. However, we found similar stromal expression of RANKL between ameloblastomas and OKCs. The apparent discrepancies between these studies may be partly due to differences in antibodies used and methods. Expression of these molecules in both the epithelial and connective tissue cells of OKCs and ameloblastomas may indicate that they play a role in the regulation of local bone metabolism.

In this study, a greater number of RANK-positive cells were seen in the epithelial component of OKCs and ameloblastomas than in radicular cysts. Since RANKL expression was uniform across all three tumor types, it would appear that the variable factor determining osteoclastogenesis is RANK expression. Taken together, these results indicate that greater bone/tooth resorption activity is present in OKCs and ameloblastomas (where RANK expression was greatest) compared to radicular cysts. Moreover, these results are consistent with the clinical behaviour of these lesions. In fact, ameloblastoma is an odontogenic neoplasm characterized by infiltration through medullary spaces with the potential to erode cortical bone [26]. Similarly, OKC may expand to cortical bone and also cause erosion, although its neoplastic nature is controversial.

In our study, we observed that the molecular system RANK/RANKL/OPG is variably expressed in OKCs, radicular cysts, and ameloblastomas by immunohistochemistry and that these mediators may be involved in the osteoclastogenetic mechanisms in OKC and ameloblastoma. Additional studies with other methods could help to clarify the role of RANK, RANKL, and OPG in mediating tumour associated bone osteolysis.

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References

1. Kurban S, Mehmetoglu I. Osteoprotegerin, RANK and RANK ligand. Turk J Biochem. 2007;34(4):178–84.
2. Stejskal D, Bartek J, Pastorková R, et al. Osteoprotegerin, RANK, RANKL. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2001;145(2):61–4.
3. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology. 2000;142(12):5050–5.

4. Kostenuik PJ, Shalhoub V. Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des. 2001;7(8):613–35.
5. Blair JM, Zheng Y, Dunstan CR. RANK ligand. Int J Biochem Cell Biol. 2007;39:1077–81.
6. Chamoux E, Houde N, L'eriger K, et al. Osteoprotegerin decreases human osteoclast apoptosis by inhibiting the TRAIL pathway. J Cell Physiol. 2008;216:536–42.
7. Boyce BF, Xing L. Function of RANKL/RANK/OPG in bone modeling end remodeling. Arch Biochem Biophys. 2008;473(2): 139–46.
8. Hofbauer LC, Neubauer A, Heufelder AE. Receptor activator of nuclear factor-Kb ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. Cancer. 2001;92(3):460–70.
9. Da Silva TA, Batista AC, Mendonça EF, et al. Comparative expression of RANK, RANKL, and OPG in keratocystic odontogenic tumors, ameloblastomas, and dentigerous cysts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008;105(3):333–41.
10. Sandra F, Hendarmin L, Kukita T, et al. Ameloblastoma induces osteoclastogenesis: a possible role of ameloblastoma in expanding in the bone. Oral Oncol. 2005;41(6):637–44.
11. Wang YP, Liu BY. High expression of osteopontin and CD44v6 in odontogenic keratocysts. J Formos Med Assoc. 2009;108(4): 286–92.
12. Kumamoto H, Ooya K. Expression of paratyroid hormone-related protein (PTHrP), osteoclast differentiation factor (ODF)/receptor activator of nuclear factor-κB ligand (RANKL) and osteoclastogenesis inhibitory factor (OCIF)/osteoprotegerin (OPG) in ameloblastomas. J Oral Pathol Med. 2004;33:46–52.
13. Shear M, Speight PM. Cysts of the oral and maxillofacial regions. 4th ed. Singapore: Blackwell Munksgaard; 2007. p. 6–58, 123–41.
14. Neville BW, Damm DD, Allen CM, Bouquot JE. Oral and maxillofacial pathology. 3th ed. Missouri: Saunders Elsevier; 2009. p. 131–35, 678–91.
15. Philipsen HP. Keratocystic odontogenic tumour. In: Barnes L, Eveson JW, Reichart P, Sinransky D, editors. Head and neck tumours, WHO classification of tumours. 5th ed. Lyon: IARC Press; 2005. p. 306–7.
16. Suda T, Takahashi N, Udagawa N, et al. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev. 1999;20(3):345–57.
17. Nakagawa N, Kinosaki M, Yamaguchi K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun. 1998;253: 395–400.
18. Wise GE, Lumpkin SJ, Huang H, et al. Osteoprotegerin and osteoclast differentiation factor in tooth eruption. J Dent Res. 2000;79(12):1937–42.
19. Rani CS, Macdougall M. Dental cells express factors that regulate bone resorption. Mol Cell Biol Res Commun. 2000;3(3):145–52.
20. Kanzaki H, Chiba M, Shimizu Y, et al. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. J Dent Res. 2001;80(3): 887–91.
21. Wada T, Nakashima T, Hiroshi N, et al. RANKL-RANK signaling in osteoclastogenesis and bone disease. Trend Mol Med. 2006;12(1):17–25.
22. Hamdy NA. Osteoprotegerin as a potential therapy for osteoporosis. Curr Osteoporos Rep. 2005;3:121–5.
23. Wittrant Y, Théoleyre S, Chipoy C, et al. RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. Biochim Biophys Acta. 2004;1704(2):49–57.
24. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. J Periodontol. 2005;76(11):2033–41.
25. Menezes R, Bramante CM, Da Silva Paiva KB, et al. Receptor activator NFkappaB-ligand and osteoprotegerin protein expression in human periapical cysts and granulomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102(3):404–9.
26. Gardner DG, Heikinheimo K, Shear M, et al. Ameloblastomas. In: Barnes L, Eveson JW, Reichart P, Sinransky D, editors. WHO classification of tumours. Pathology and genetics of head and neck tumour. 5th ed. Lyon: IARC Press; 2005. p. 296–300.