Immunohistochemical Detection of Receptor Activator Nuclear κB Ligand and Osteoprotegerin in Odontogenic Cysts and Tumors

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Purpose: The aim of the present study was to compare the immunohistochemical detection of receptor activator nuclear κB ligand (RANKL) and osteoprotegerin (OPG) in radicular cysts (RCs), dentigerous cysts (DCs), solid ameloblastomas (SAs), and keratocystic odontogenic tumors (KOTs).

Materials and Methods: A total of 20 RCs, 20 DCs, 20 KOTs, 14 dental follicles (DFs), and 18 SAs were evaluated by immunohistochemistry using anti-RANKL and anti-OPG antibodies. The analysis was quantitative, and the number of positive cells was counted in 10 microscopic high-power fields (400×).

Results: The DFs, KOTs, and SAs showed higher expression of RANKL than did the RCs and DCs in the epithelium (P < .05). The epithelial expression of OPG was higher in the DFs, KOTs, RCs, and DCs than in the SAs (P < .05). The ratio of OPG less than RANKL was more frequent in SAs and OPG greater than RANKL in DCs (P < .05).

Conclusions: Our results have shown differences in RANKL and OPG detection in the odontogenic cysts and tumors studied. The higher RANKL and lower OPG detection in SA could play a role in bone resorption, compatible with the tumor’s biologic behavior.

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Odontogenic cysts and tumors constitute a heterogeneous group of jaw lesions with diverse histopathologic features and clinical manifestations. Bone resorption is a major biologic event responsible for the progression and aggressiveness of these lesions and depends on the formation and activation of osteoclasts. The expression of factors stimulating and inhibiting bone resorption has been correlated with the development of these lesions, with emphasis on the osteoprotegerin (OPG) known as osteoclastogenesis inhibitory factor and receptor activator of nuclear factor κB ligand (RANKL), an osteolytic factor.

It is known that the balance between bone resorption and formation is required under normal physiologic conditions for bone homeostasis, promoting the maintenance of structural integrity of the tissue. A new perspective in the understanding at the molecular level regarding osteoclast biology and bone homeostasis has emerged with the identification of the triad receptor activator of nuclear factor κB (RANK), its ligand RANKL, and OPG.
The upregulation of RANKL and the downregulation of OPG have been implicated in a range of diseases, including bone malignancies such as intraosseous oral squamous cell carcinoma. Moreover, they might be involved in regulation of the immune system, arterial calcification, and a number of metabolic bone diseases. Some studies have reported conflicting results. Greater expression of RANKL was found in odontogenic tumors (OTs), such as the calcifying epithelial OT, ameloblastic fibroma, and odontogenic myxoma. However, other studies have reported higher and lower RANKL expression for the keratocystic OT (KOT), similar to ameloblastoma, with both higher and lower RANKL expression reported.

Nevertheless, some studies have investigated the immunohistochemical expression of these proteins. Our study evaluated odontogenic inflammatory and developmental cysts, aggressive odontogenic tumors, and dental follicles (DFs), which have indolent behavior. The aim of the present study was to compare the expression of RANKL and OPG in samples of radicular cysts (RCs), dentigerous cysts (DCs), solid ameloblastomas (SAs), and KOTs, odontogenic lesions with different biologic behavior.

**Materials and Methods**

The ethics committee of the Federal University of Rio Grande do Norte approved the present research (protocol no. 175/2010).

**SAMPLES**

The original hematoxylin and eosin–stained slides and formalin-fixed, paraffin-embedded specimen blocks of 20 RCs, 20 DCs, 18 SAs, 20 KOTs, and 14 DFs were retrieved from the laboratory archives. The diagnosis of the cysts was determined mainly from the radiographic and histopathologic examination findings and that of the odontogenic tumors (ie, SA and KOT) was in accordance with previously described morphologic criteria. Intensely inflamed DCs were excluded, and DFs were included as controls of non-neoplastic odontogenic epithelium. The distinction between DCs and DFs was made using microscopic, clinical and surgical, and radiographic findings. A DC was predominantly lined by stratified squamous epithelium, and surgical exploration revealed bone cavitation and cystic content. A DF was predominantly lined by reduced enamel epithelium, and no bone cavitation or cystic content was detected.

**IMMUNOHISTOCHEMISTRY**

For immunohistochemical analysis, 3-μm-thick, paraffin-embedded tissue sections were placed on 3-aminopropyltriethoxisilane coated glass slides (Sigma-Aldrich, St Louis, MO). The samples were deparaffinized with xylene, rehydrated in graded alcohols, and washed in deionized water and phosphate-buffered saline. The samples were then incubated with 3% hydrogen peroxide and immersed in a citrate buffer (pH 6.0) for 3 minutes at 121°C. The sections were then blocked by incubation with 3% normal goat serum at room temperature for 20 minutes, and the slides were incubated at 4°C, overnight, in a humidified chamber with the following primary rabbit polyclonal antibodies: anti-OPG (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200; and anti-RANKL (N-19; Santa Cruz Biotechnology) diluted 1:200. After washing in Tris-buffered saline, the sections were treated with

| Table 1. PARAMETERS USED FOR CALCULATION OF KRUSKAL-WALLIS TEST TO EVALUATE SCORES OF CELLS IMMUNOSTAINED BY RANKL AND OPG IN LINING EPITHELIUM STRATIFIED BY LESION TYPE |
|---------------- |------ |------- |---------------- |---------------- |---------------- |
| Immunostain     | Group | n     | Median | 25th to 75th Quartile | Mean of Ranks |
| RANKL           | RC    | 20    | 2.00   | 1.00-3.00 | 35.50 |
|                 | DC    | 20    | 2.00   | 2.00-3.00 | 38.50 |
|                 | DF    | 14    | 3.00   | 3.00-3.00 | 63.00 |
|                 | SA    | 18    | 3.00   | 2.00-3.00 | 50.33 |
|                 | KOT   | 20    | 3.00   | 2.00-3.00 | 45.00 |
| OPG             | RC    | 20    | 3.00   | 2.00-3.00 | 44.00 |
|                 | DC    | 20    | 3.00   | 3.00-3.00 | 49.15 |
|                 | DF    | 14    | 3.00   | 3.00-3.00 | 53.65 |
|                 | SA    | 18    | 2.00   | 1.00-3.00 | 27.50 |
|                 | KOT   | 20    | 3.00   | 3.00-3.00 | 51.08 |

Abbreviations: DC, dentigerous cyst; DF, dental follicle; KOT, keratocystic odontogenic tumor; OPG, osteoprotegerin; RANKL, receptor nuclear factor κB ligand; RC, radicular cyst; SA, solid ameloblastoma.

* Kruskal-Wallis test.

a labeled streptavidin-biotin kit (Dako, Glostrup, Denmark). Peroxidase activity was visualized by immersing the tissue sections in 3,3′-diaminobenzidine (D5637; Sigma Aldrich) and counterstained with Mayer’s hematoxylin. A central giant cell granuloma was used as a positive control. Negative controls were obtained by the omission of the primary antibodies and substitution of the primary antibodies with nonimmune rabbit serum (X0902; Dako).

**CELL COUNTING**

The number of immunopositive cells for RANKL and OPG was evaluated in epithelium/parenchyma and fibrous capsule/stroma by 2 independent examiners. The analysis was quantitative, and the number of positive cells was counted in 10 representative and consecutive microscopic high-power fields (400×) over totally counted cells, irrespective of the cell type. Digital images were loaded on IMAGE J (National Institutes of Health, Bethesda, MD) to count the number of immunostained cells, and the percentage of positive cells was classified according as follows: 0 or no staining, less than 10% positive immunostaining cells; 1 or weak staining, 11% to 25%; 2 or moderate staining, 26% to 75%; and 3 or strong staining, greater than 76%.9

**STATISTICAL ANALYSIS**

A comparative analysis of the data was performed using the nonparametric Wilcoxon signed rank test and the Kruskal-Wallis test. Statistical significance was set at \( P \leq .05 \).

**Results**

**QUALITATIVE AND QUANTITATIVE ANALYSIS OF EPITHELIAL AND PARENCHYMAL CELLS**

Immunohistochemical reactivity for RANKL and OPG was detected in the nuclei and cytoplasm of cellular components in neoplastic and non-neoplastic epithelium. Statistical differences were observed in cell reactivity in the lining epithelium and parenchyma of the lesions \( (P < .05; \text{Table 1}) \). A higher expression of RANKL was found in the SA, KOT and DF \( (\text{Fig 1A, 1B and 1E}) \) than in the DC and RC \( (\text{Fig 1C and 1D}) \). Regarding the expression of OPG, a greater number of immunopositive cells were noted in the KOT, DC, RC and DF \( (\text{Fig 2B, 2C, 2D and 2E}) \) than in the SA \( (\text{Fig 2A}) \).

In addition, significant differences were observed in the distribution of cases with respect to the OPG/RANKL ratio of immunostaining scores in the lining epithelium. Most cases of RC (50%), DC (55%), and KOT (60%) exhibited a similar content of OPG and RANKL \( (P < .05) \). However, 50% of the SAs exhibited less OPG than RANKL \( (\text{Table 1}) \).

**QUALITATIVE AND QUANTITATIVE ANALYSIS OF FIBROUS CAPSULE AND STROMA**

Regarding the reactivity for RANKL and OPG in the fibrous capsule and stromal cells, positive fibroblasts, endothelial cells, polymorphonuclear neutrophils, plasmocytes, lymphocytes, and macrophage cells were observed. Additionally, RANKL and OPG expression was observed in nests of odontogenic epithelial cells.

The quantitative analysis of the lesions immunostained for RANKL and OPG in the fibrous capsule and stroma is summarized in Table 2. Statistically, differences were observed in cell reactivity for RANKL.

![Image](image-url)
and OPG between the odontogenic cysts and the tumors (Table 2). Higher expression of RANKL was observed in DF and SA than in KOT, DC, and RC ($P < .05$). OPG expression was greater in DF and KOT than in DC, RC, and SA ($P < .05$).

For the OPG/RANKL ratio, significant differences were observed in the distribution of cases with respect to the scores in the fibrous capsule and stroma. We observed that most cases of RC (50%) exhibited similar content of OPG and RANKL ($P < .05$), and most cases of SA (72.2%) exhibited less OPG than RANKL ($P < .05$; Table 3).

No positive staining was observed when the primary antibodies were omitted. The positive control samples showed strong reactivity.

**Discussion**

Our results have demonstrated differences in RANKL and OPG detection in these lesions. 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.11,12 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.13,14

Ameloblastomas and KOTs are lesions with aggressive clinical behavior, a variety of morphologic patterns, and high recurrence rates.15,16 Because these tumors generally exhibit distinct radiographic characteristics of bone and tooth resorption, a differential content of RANKL and OPG can be expected in these lesions compared with odontogenic cysts such as RCs and DCs.

In accordance with this, we noted a higher number of RANKL-positive cells in the parenchyma of the SA and KOT specimens compared with the lining epithelium of the DCs and RCs. Increased expression of RANKL would be related to increased osteoclast activity, thus favoring bone resorption.17,18 However, KOTs...
and DCs had a higher number of OPG-positive cells in the parenchyma and lining epithelium and stroma and fibrous capsule cells compared with the SAs and RCs. This lower OPG immunodetection for SAs and RCs suggests inhibition of osteolytic activity, because the presence of this protein would block the interaction of RANK and RANKL, inhibiting the terminal stage of osteoclast differentiation, thereby resulting in decreased bone resorption.18,19

Menezes et al20 demonstrated a higher secretion of RANKL under inflammatory conditions. This increased secretion would not always guide the bone resorption process, because the presence of the inhibitory receptor OPG would decisively influence the process of bone expansion. Furthermore, other bone metabolism-related factors could also be involved in this complex process, such as macrophage colony-stimulating factor and tumor necrosis factor-α. It has also been shown that some components of the NF-κB pathway are involved in tooth development, including RANK, RANKL, and OPG.21 Ohazama et al21 observed the expression patterns of RANKL and OPG during odontogenesis. RANKL was expressed in the tooth mesenchyme and OPG in the tooth epithelium and mesenchyme. Thus, it was interesting to find that DFs exhibited a greater number of RANKL- and OPG-positive cells in the lining epithelium and fibrous capsule. These proteins would be in equilibrium, justifying their indolent biologic behavior.

FIGURE 2. A, Solid ameloblastoma with weak osteoprotegerin (OPG) expression in parenchyma and stromal cells (LSAB, ×400). B, Keratocystic odontogenic tumor with strong OPG expression in parenchyma and stromal cells (LSAB, ×400).


FIGURE 2 (cont’d). C, Dentigerous cyst with strong OPG expression in lining epithelium and fibrous capsule (LSAB, ×400). D, Radicular cyst with strong OPG expression in lining epithelium and fibrous capsule (LSAB, ×400).


FIGURE 2 (cont’d). E, Dental follicle with strong OPG expression in lining epithelium and fibrous capsule (LSAB, ×400).

da Silva et al. observed no difference in the expression of RANKL among SAs, KOTs, DCs, and DFs in the parenchyma and lining epithelium. They reported that neoplastic and non-neoplastic odontogenic epithelial cells seemed to be an important source of RANKL and OPG. In the stromal cells, they verified higher immunoexpression of OPG in KOTs, SAs, and DCs than in DFs and higher RANKL expression in SAs and KOTs than in the DFs. Moreover, they noted higher expression of RANKL in SAs than in DCs in the stromal cells. Their results are consistent with the clinical behavior of these lesions (SAs and DCs).

Tekkesin et al. verified a similar expression of RANKL among SAs, KOTs, and RCs in the odontogenic epithelium; however, they found higher stromal expression of RANKL in RCs than in SAs. With respect to OPG expression, they observed a lower number of positive cells in KOTs, SAs, and RCs in the epithelium and parenchyma and stroma and fibrous capsule, but they noted higher expression in SAs and RCs than in KOTs in the stromal cells. The apparent discrepancies between these studies could be explained in part by the differences in antibodies used and/or method of quantification. The expression of RANKL

### Table 2. PARAMETERS USED FOR CALCULATION OF KRUSKAL-WALLIS TEST TO EVALUATE SCORES OF CELLS IMMUNOSTAINED BY RANKL AND OPG IN FIBROUS CAPSULE STRATIFIED BY LESION TYPE

<table>
<thead>
<tr>
<th>Immunostain</th>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>25th to 75th Quartile</th>
<th>Mean of Ranks</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANKL</td>
<td>RC</td>
<td>20</td>
<td>2.00</td>
<td>2.00-3.00</td>
<td>36.20</td>
<td>.007</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>20</td>
<td>3.00</td>
<td>1.25-3.00</td>
<td>39.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>14</td>
<td>3.00</td>
<td>3.00-3.00</td>
<td>59.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>18</td>
<td>3.00</td>
<td>3.00-3.00</td>
<td>54.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOT</td>
<td>20</td>
<td>3.00</td>
<td>2.00-3.00</td>
<td>41.05</td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>RC</td>
<td>20</td>
<td>2.00</td>
<td>1.00-3.00</td>
<td>40.73</td>
<td>.019</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>20</td>
<td>2.00</td>
<td>1.25-3.00</td>
<td>44.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>14</td>
<td>3.00</td>
<td>3.00-3.00</td>
<td>64.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>18</td>
<td>2.00</td>
<td>1.00-2.25</td>
<td>33.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KOT</td>
<td>20</td>
<td>3.00</td>
<td>1.00-3.00</td>
<td>48.30</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DC, dentigerous cyst; DF, dental follicle; KOT, keratocystic odontogenic tumor; OPG, osteoprotegerin; RANKL, receptor nuclear factor κB ligand; RC, radicular cyst; SA, solid ameloblastoma.

* Kruskal-Wallis test.


### Table 3. DISTRIBUTION OF CASES ACCORDING TO OPG AND RANKL IMMUNOSTAINING SCORES IN EPITHELIUM AND FIBROUS CAPSULE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lesion Type</th>
<th>OPG &lt; RANKL</th>
<th>OPG &gt; RANKL</th>
<th>OPG = RANKL</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium</td>
<td>RC</td>
<td>2 (10)</td>
<td>8 (40)</td>
<td>10 (50)</td>
<td>.031</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>0 (0)</td>
<td>9 (45)</td>
<td>11 (55)</td>
<td>.004</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>3 (21.4)</td>
<td>0 (0)</td>
<td>11 (78.6)</td>
<td>.317</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>9 (50)</td>
<td>0 (0)</td>
<td>9 (50)</td>
<td>.006</td>
</tr>
<tr>
<td></td>
<td>KOT</td>
<td>1 (5)</td>
<td>7 (35)</td>
<td>12 (60)</td>
<td>.054</td>
</tr>
<tr>
<td>Capsule/stroma</td>
<td>RC</td>
<td>8 (40)</td>
<td>2 (10)</td>
<td>10 (50)</td>
<td>.050</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>6 (30)</td>
<td>2 (10)</td>
<td>12 (60)</td>
<td>.473</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>3 (21.4)</td>
<td>0 (0)</td>
<td>11 (78.6)</td>
<td>.317</td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>13 (72.2)</td>
<td>0 (0)</td>
<td>5 (27.8)</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>KOT</td>
<td>6 (30)</td>
<td>4 (20)</td>
<td>10 (50)</td>
<td>.158</td>
</tr>
</tbody>
</table>

Data presented as n (%).

Abbreviations: DC, dentigerous cyst; DF, dental follicle; KOT, keratocystic odontogenic tumor; OPG, osteoprotegerin; RANKL, receptor nuclear factor κB ligand; RC, radicular cyst; SA, solid ameloblastoma.

* Nonparametric Wilcoxon signed rank test.

RANKL and OPG Detection in Cysts and Tumors

in both epithelial and mesenchymal cells of SAs, KOTs, DCS, and RCs could indicate that they play a role in the regulation of local bone metabolism.²

Among the odontogenic cysts, we found a higher number of RANKL-positive cells in the lining epithelium and capsule of DCS than in RCs. According to de Moraes et al,⁹ these findings are compatible with the greater expansive potential of DCS by indicating the presence of a larger number of osteoclast precursors expressing RANK that are able to interact with their specific receptors (RANKL), leading to osteoclast differentiation and maturation. The expression in nests of odontogenic epithelial cells could also have contributed to this higher expression in the fibrous capsule of DCS.

Additionally, we verified the OPG/RANKL ratio in the epithelium and parenchyma and fibrous capsule and stroma of odontogenic cysts and tumors. The amount of OPG and RANKL was equal in most cases; however, for SA, OPG expression was less than RANKL expression, which could indicate osteolytic activity. Qian and Huang¹⁵ reported OPG suppressed both the osteoclastogenesis induced by ameloblastoma cells and the bone resorption caused by osteoclasts. These investigators suggested OPG could be used for therapeutic application in ameloblastomas in the foreseeable future. Sathi et al²² suggested that the stroma not only acts in bone resorption, but also in the suppression of new bone formation in SA.

Despite the higher number of DF cells immunopositive for RANKL and OPG, the ratio RANKL/OPG was consistently around 1, which is compatible with the indolent behavior shown by the entity. OTs having cystic architecture, but with a neoplastic nature (eg, KOT) have a tendency to present with a smaller RANKL/OPG cell ratio than do solid lesions.¹ Taken together, it seems plausible to suggest that higher immunodetection of RANKL and lower immunodetection of OPG could indicate greater bone and tooth resorption activity in KOTs and SAs compared with RCs and DCS. This is consistent with the clinical behavior of these lesions. SA is an odontogenic neoplasm characterized by infiltration through the medullary spaces, with the potential to erode cortical bone, similar to KOT, which can expand to cortical bone and also cause erosion, although its neoplastic nature has been controversial.²³

The present findings have shown differences in RANKL and OPG immunodetection among the lesions studied. The higher RANKL and lower OPG expression in SA could play a role in bone resorption, compatible with the tumor’s biologic behavior.

References