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Immunohistochemical analysis of bone resorption regulators (RANKL and OPG), angiogenic index, and myofibroblasts in syndrome and non-syndrome odontogenic keratocysts

Cassiano Francisco Weege Nonaka [a], *Roberta Barroso Cavalcante* [b], *Renato Luiz Maia Nogueira* [c], *Lélia Batista de Souza* [a], *Leão Pereira Pinto* [a,*]

[a] *Department of Oral Pathology, Federal University of Rio Grande do Norte, Natal, RN, Brazil*
[b] *Department of Oral Pathology, University of Fortaleza, Fortaleza, CE, Brazil*
[c] *Department of Oral Surgery, Federal University of Ceará, Fortaleza, CE, Brazil*

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ABSTRACT

Objective: The aim of this study was to immunohistochemically analyse bone resorption regulators (receptor activator of nuclear factor kappa B ligand [RANKL] and osteoprotegerin [OPG]), angiogenic index, and myofibroblasts in Gorlin syndrome-related odontogenic keratocysts (SOKCs) and non-syndrome odontogenic keratocysts (NSOKCs).

Study design: Twenty-two SOKCs, 22 primary NSOKCs, and eight recurrent NSOKCs were evaluated by immunohistochemistry using anti-RANKL and anti-OPG antibodies. The angiogenic index was determined by microvessel count (MVC) using anti-CD34 antibody. Anti-α-smooth muscle actin (α-SMA) antibody was used for the identification of myofibroblasts.

Results: Analysis of the expression of RANKL and OPG in the epithelial lining and fibrous capsule did not reveal significant differences between groups ($P > 0.05$). In the epithelial lining, the RANKL/OPG ratio was RANKL < OPG and RANKL = OPG in most primary NSOCKs (54.5%) and SOKCs (59.1%), respectively ($P > 0.05$). In the fibrous capsule, the ratio was RANKL = OPG in most primary (81.8%) and recurrent NSOKCs (75.0%) and in most SOKCs (45.5%) ($P > 0.05$). No significant differences in the angiogenic index or number of myofibroblasts were observed between primary NSOKCs, recurrent NSOKCs, and SOKCs ($P > 0.05$).

Conclusions: The present results suggest that differences in the biological behaviour of SOKCs and NSOKCs may not be related to the expression of RANKL and OPG, to the RANKL/OPG ratio, to the angiogenic index, or to the number of myofibroblasts in these lesions.

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1. Introduction

Odontogenic keratocysts (OKCs), which have been recently reclassified by the World Health Organization as benign neoplasms,[1] are distinguished from other odontogenic cysts by their aggressive biological behaviour, tendency towards recurrence, and association with nevoid basal cell carcinoma syndrome or Gorlin syndrome.[2,3]

OKCs associated with Gorlin syndrome (SOKCs) have been suggested to present a greater growth and infiltration capacity and a higher tendency to recur than non-syndrome OKCs

* *Corresponding author at*: Universidade Federal do Rio Grande do Norte, Departamento de Odontologia, Av. Senador Salgado Filho, 1787, Lagoa Nova, Natal, RN, Brasil, CEP 59056-000. Tel.: +55 84 3215 4138; fax: +55 84 3215 4138.
E-mail address: leao.p.pinto@gmail.com (L.P. Pinto).

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(NSOKCs).[4–6] The results of studies on cell cycle and apoptosis,[4,7] oncogenes, tumour suppressor genes,[7] extracellular matrix composition,[8] and proteases[9,10] support the existence of a distinct biological behaviour of SOKCs and NSOKCs.Recent reports have shown that proteins involved in bone remodeling[11,12] and angiogenesis,[13] as well as myofibroblasts,[14,15] play an important role in the growth and progression of odontogenic cysts and tumours. According to some of these studies, the expression of proteins involved in bone resorption, the angiogenic index, and the number of myofibroblasts tend to be higher in lesions with a more aggressive biological behaviour.[12–14]

Bone remodelling is a highly coordinated process that involves bone resorption mediated by osteoclasts and synthesis of organic matrix mediated by osteoblasts.[16,17] Particularly, three molecules belonging to the tumour necrosis factor ligand and receptor superfamilies, called receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG), are critical for the control of bone remodelling[17,18] and also play a potential secondary role in angiogenesis.[19,20]

Angiogenesis is the formation of new blood vessels from the preexisting vasculature,[21] and it can be evaluated by the quantification of vessels immunolabeled with antibodies against endothelial cell epitopes such as CD34.[13] The importance of vascular networks for the development and maintenance of tissues has been demonstrated in many physiological and pathological processes such as wound healing, arthritis, and tumour progression.[22,23]

Myofibroblasts are specialised cells that show a hybrid phenotype between fibroblasts and smooth muscle cells.[15] Due to their contractile features and capacity to synthesise extracellular matrix components, myofibroblasts have been implicated in the pathogenesis of fibrocontractive diseases such as renal fibrosis.[24,25] In addition, myofibroblasts seem to play a role in tumour progression due to their ability to secrete proteases and cytokines.[25,26]

In light of these findings, the objective of the present study was to immunohistochemically analyse bone resorption regulators (RANKL and OPG), angiogenic index, and myofibroblasts in SOKCs and NSOKCs in order to better understand the differences in the biological behaviour of these lesions.

2. Materials and methods

Fifty-two OKC specimens, including 22 SOKCs, 22 primary NSOKCs and 8 recurrent NSOKCs, obtained from the Oral Pathology Departments of the Federal University of Rio Grande do Norte (UFRN) and of the University of Fortaleza (UNIFOR), were randomly selected for this study. The size of the sample was defined by the number of available institutional archival cases. In all cases, the histological diagnosis was based on the second WHO classification.[27] All syndrome patients had been diagnosed according to the criteria proposed by Evans et al.[28] and presented multiple OKCs. The patients with sporadic OKCs had single lesions and had been submitted to clinical and radiographical assessment to exclude the presence of other manifestations of Gorlin syndrome. Serial 3-μm thick sections were cut from the tissue blocks and processed for immunohistochemical examination. The study was approved by the Research Ethics Committee of UFRN, Natal, Brazil.

2.1. *Immunohistochemistry*

The tissue sections were deparaffinised and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then washed in phosphate-buffered saline (PBS). Antigen retrieval, antibody dilution and clone type for RANKL, OPG, CD34 and α-SMA are shown in Table 1. After treatment with normal serum, the sections were incubated with the primary antibodies in a moist chamber. The sections were then washed twice in PBS and treated with the labelled streptavidin biotin complex (LSAB + System-HRP; Dako, Carpinteria, CA) at room temperature to bind the primary antibodies. Peroxidase activity was visualised by immersing the tissue sections in diaminobenzidine (Liquid DAB + ; Dako, Carpinteria, CA), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's haematoxylin and coverslipped. Sections of central giant cell lesion served as positive control samples for RANKL and OPG. Sections of lobular capillary hemangioma were used as positive control for CD34 and α-SMA. Samples treated as described above, except that the primary antibody was replaced with a solution of bovine serum albumin in PBS, were used as negative control.

2.2. *Analysis of immunostaining*

The tissue sections were examined in a blind fashion by two observers under an Olympus CX31 light microscope (Olympus Japan Co., Tokyo, Japan). The immunoexpression of RANKL and OPG was evaluated both in the epithelial lining and in the fibrous capsule of OKCs. In the epithelial lining, immunopositive cells were evaluated semiquantitatively according to an adaptation of the method used by Nonaka et al.[29] The immunoexpression of RANKL and OPG was classified as follows at ×100 magnification: 0 (≤10% immunopositive cells), 1 (11–50% immunopositive cells), 2 (51–75% immunopositive cells), and 3 (>75% immunopositive cells). Next, each case was assigned to one of the following groups according to the

Table 1 – Clone, specificity, manufacturer, dilution, antigen retrieval and incubation of the primary antibodies.

Clone	Specificity	Manufacturer	Dilution	Antigen retrieval	Incubation
N-19	RANKL	Santa Cruz Biotechnology, Santa Cruz, CA	1:200	Citrate, pH 6.0, Pascal, 3 min	18 h
N-20	OPG	Santa Cruz Biotechnology, Santa Cruz, CA	1:200	Citrate, pH 6.0, Pascal, 3 min	18 h
α-sm1	α-SMA	Novocastra Laboratories, Benton Lane, NET	1:50	Citrate, pH 6.0, Pascal, 3 min	60 min
QBEnd-10	CD34	Dako, Carpinteria, CA	1:50	Tris–EDTA, pH 9.0, Pascal, 3 min	18 h

RANKL/OPG ratio: RANKL > OPG, RANKL < OPG, and RANKL = OPG.

The immunoexpression of RANKL and OPG in the fibrous capsule of OKCs was evaluated quantitatively. An adaptation of the method proposed by da Silva et al.[12] was used. Tissue sections were examined by light microscopy to identify five fields immediately beneath the cystic epithelial lining that contained the largest number of immunostained cells. The number of positive and negative cells was determined in each field at ×400 magnification, permitting the calculation of the percentage of RANKL- and OPG-positive cells in each case. On the basis of this percentage, the following scores were assigned to each sample: 0 (≤10% immunostained cells), 1 (11–50% immunostained cells), 2 (51–75% immunostained cells), and 3 (>75% immunostained cells).[29] Next, each case was assigned to one of the following groups according to the RANKL/OPG ratio: RANKL > OPG, RANKL < OPG, and RANKL = OPG.

The angiogenic index in SOKCs and NSOKCs was determined based on the number of anti-CD34-immunoreactive vessels. Microvessel count (MVC) was performed as described by Maeda et al.[30] Tissue sections were examined by light microscopy at ×100 magnification and five areas immediately beneath the cystic epithelial lining showing the highest vascularisation were identified. Vessels were counted in these five areas of highest vascular density at ×200 magnification. MVC is expressed as the mean number of vessels in these areas per sample. Single endothelial cells or clusters of these cells, with or without lumen, were considered to be individual vessels.

With respect to myofibroblasts, α-SMA-immunopositive cells were quantified according to an adaptation of the method proposed by Vered et al.[14] Tissue sections were examined by light microscopy at ×100 magnification to identify 10 fields immediately beneath the cystic epithelial lining that contained the largest number of immunostained cells. In these fields, α-SMA-positive cells, excluding those surrounding blood vessels, were counted at ×400 magnification and the total number of positive cells in all 10 fields examined per case was calculated, permitting the calculation of the mean number of α-SMA-positive cells per field.

2.3. Statistical analysis

The results obtained were submitted to statistical analysis using the Statistical Package for the Social Sciences (version 17.0; SPSS Inc., Chicago, IL). The nonparametric Kruskal–Wallis test was applied to compare the percentage of RANKL- and OPG-immunopositive cells in the epithelial lining and in the fibrous capsule between SOKCs, primary NSOKCs, and recurrent NSOKCs. The chi-square test was used to analyse the RANKL/OPG ratio in the epithelial lining and fibrous capsule of SOKCs, primary NSOKCs, and recurrent NSOKCs. MVC and the number of myofibroblasts was compared between SOKCs, primary NSOKCs and recurrent NSOKCs by the nonparametric Kruskal–Wallis test. Spearman's correlation test was performed to determine possible correlations between MVC, number of myofibroblasts, and percentage of RANKL- and OPG-immunopositive cells. For all tests, significance level was set at 5% ($P < 0.05$).

3. Results

3.1. Immunoexpression of RANKL and OPG

Analysis of the expression of RANKL and OPG in the epithelial lining of OKCs revealed cytoplasmic immunoreactivity for these proteins, with no peculiar pattern of distribution in the different cell layers. For RANKL, most SOKCs were classified as score 3 (63.6%) (Fig. 1a), followed by scores 1 (27.3%) and 2 (9.1%). In primary NSOKCs, most cases were classified as score 3 (40.9%), followed by scores 2 (36.4%) and 1 (22.7%) (Fig. 1b). With respect to recurrent NSOKCs, there was a slight predominance of cases scored as 2 (50.0%), followed by scores 3 (25.0%) and 1 (25.0%). Statistical analysis revealed no significant differences between groups ($P = 0.408$).

Regarding the epithelial expression of OPG, SOKCs showed a predominance of cases scored as 3 (81.8%), followed by scores 2 (9.1%), 1 (4.5%) (Fig. 1c), and 0 (4.5%). In primary NSOKCs, most cases were classified as score 3 (86.4%) (Fig. 1d), followed by scores 2 (9.1%) and 1 (4.5%). In recurrent NSOKCs, there was a predominance of cases scored as 3 (62.5%), followed by scores 1 (25.0%) and 2 (12.5%). The nonparametric Kruskal–Wallis test showed no significant differences between groups ($P = 0.313$).

With respect to the RANKL/OPG ratio in the epithelial lining, a RANKL < OPG ratio was observed in most primary NSOKCs (54.5%), whereas most SOKCs presented a RANKL = OPG ratio (59.1%). Regarding recurrent NSOKCs, there was a slight predominance of cases with a RANKL < OPG ratio (50.0%). Statistical analysis revealed no significant differences between groups ($P = 0.573$) (Table 2).

In the fibrous capsule, RANKL and OPG were expressed in endothelial cells, in fibroblasts (Fig. 1a–d), and in bone surrounding cells. With respect to the expression of RANKL, most primary NSOKCs were scored as 3 (54.5%), followed by scores 2 (40.9%) and 1 (4.5%). In recurrent NSOKCs, most cases were classified as score 2 (62.5%), followed by score 3 (37.5%). In SOKCs, there was a predominance of cases scored as 3 (54.5%), followed by scores 2 (36.4%) and 1 (9.1%). The nonparametric Kruskal–Wallis test showed no significant differences between groups ($P = 0.805$).

Regarding the immunoexpression of OPG in the fibrous capsule, primary NSOKCs were predominantly classified as score 3 (63.6%), followed by scores 2 (31.8%) and 1 (4.5%). In recurrent NSOKCs, most cases were classified as score 2 (62.5%), followed by scores 3 (25.0%) and 1 (12.5%). With respect to SOKCs, there was a slight predominance of cases scored as 3 (59.1%), followed by score 2 (40.9%). Statistical analysis revealed no significant differences between groups ($P = 0.128$).

Analysis of the RANKL/OPG ratio in the fibrous capsule of primary NSOKCs revealed a predominance of RANKL = OPG (81.8%). Similarly, most recurrent NSOKCs (75.0%) and SOKCs (45.5%) showed a RANKL = OPG ratio. No statistically significant differences were observed between groups ($P = 0.061$) (Table 2).

3.2. Angiogenic index

Immunohistochemical reactivity for CD34 was detected in endothelial cells of vessels with distinct lumens, as well as in

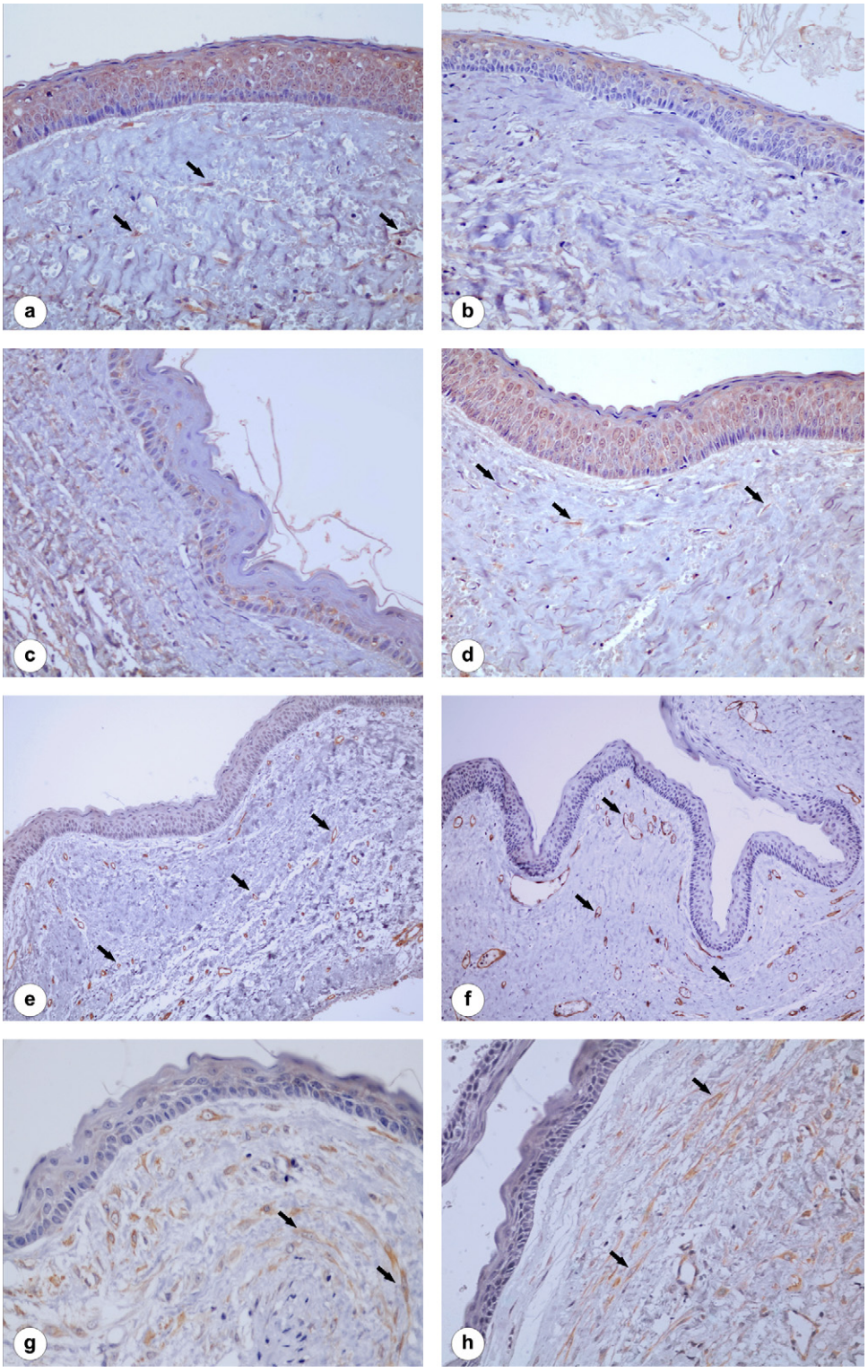

Fig. 1 – (a) Expression of RANKL in the epithelial lining (score 3) and in cells (arrows) of the fibrous capsule of SOKC (LSAB, ×400). (b) Expression of RANKL in the epithelial lining (score 1) of primary NSOKC (LSAB, ×400). (c) Expression of OPG in the epithelial lining (score 1) of SOKC (LSAB, ×400). (d) Expression of OPG in the epithelial lining (score 3) and in cells (arrows) of the fibrous capsule of primary NSOKC (LSAB, ×400). (e) Vessels immunoreactive to anti-CD34 antibody (arrows) in SOKC, most of them exhibiting small lumen (LSAB, ×100). (f) Variably sized vessels immunoreactive to anti-CD34 antibody (arrows) in recurrent NSOKC (LSAB, ×100). (g) Presence of myofibroblasts (arrows) in the fibrous capsule of SOKC (LSAB, ×400). (h) Presence of myofibroblasts (arrows) in the fibrous capsule of primary NSOKC (LSAB, ×400).

Table 2 – Distribution of cases of primary non-syndrome OKCs, recurrent non-syndrome OKCs, and syndrome OKCs according to the RANK/OPG ratio in the epithelial lining and in the fibrous capsule.

Location/group	RANKL/OPG ratio			P
	RANKL > OPG n (%)	RANKL < OPG n (%)	RANKL = OPG n (%)	
Epithelial lining				
Primary non-syndrome OKC	1 (4.5)	12 (54.5)	9 (40.9)	0.573
Recurrent non-syndrome OKC	1 (12.5)	4 (50.0)	3 (37.5)	
Syndrome OKC	2 (9.1)	7 (31.8)	13 (59.1)	
Fibrous capsule				
Primary non-syndrome OKC	1 (4.5)	3 (13.6)	18 (81.8)	0.061
Recurrent non-syndrome OKC	2 (25.0)	0 (0.0)	6 (75.0)	
Syndrome OKC	5 (22.7)	7 (31.8)	10 (45.5)	

single endothelial cells and clusters of these cells without lumen. The mean number of blood vessels determined by MVC was 71.6 (range: 52.4–107.2) in SOKCs (Fig. 1e) and 69.2 (range: 47.0–108.0) in primary NSOKCs. In recurrent NSOKCs, the mean number of blood vessels was 67.6 (range: 34.0–104.4) (Fig. 1f). The nonparametric Kruskal–Wallis test revealed no significant differences between groups ($P = 0.809$).

Spearman's correlation test revealed no significant correlation between the angiogenic index and the expression of RANKL or OPG in the epithelial lining ($P > 0.05$) and fibrous capsule ($P > 0.05$) of all groups of OKCs.

3.3. *Myofibroblasts*

Analysis of the immunohistochemical expression of anti-α-SMA antibody showed the presence of myofibroblasts in all groups of OKCs. The mean number of myofibroblasts was 33.7 (range: 20.5–56.7) in SOKCs (Fig. 1g) and 34.4 (range: 17.9–53.2) in primary NSOKCs (Fig. 1h). In recurrent NSOKCs, the mean number of myofibroblasts was 29.3 (range: 11.7–44.6). The nonparametric Kruskal–Wallis test showed no significant differences between groups ($P = 0.590$).

For all groups, Spearman's correlation test showed no significant correlation between the number of myofibroblasts and the angiogenic index ($P > 0.05$).

4. Discussion

SOKCs and NSOKCs have been suggested to share a common pathogenesis, which is characterised by mutations in the *PTCH* gene and consequent aberrant activation of the SHH signalling pathway.[31,32] Despite these studies, SOKCs have been reported to present a greater growth and infiltration capacity[4] and a higher tendency to recur[5,6] than NSOKCs. Accordingly, the results of several investigations support the existence of a distinct biological behaviour of SOKCs and NSOKCs.[4,7–10]

The molecular triad RANK, RANKL and OPG is critical for the control of osteoclastogenesis and pathophysiological bone remodelling.[18] Binding of RANKL to RANK leads to preosteoclast recruitment, fusion into multinucleate osteoclasts, osteoclast activation, and osteoclast survival.[17] On the other hand, OPG suppresses bone resorption by inhibiting the interaction between RANK and RANKL.[17,18] Alterations in the balance between RANKL and OPG play a pivotal role in several osteolytic diseases[18,33] and may be due to an increase in the concentration of RANKL or a decrease in the concentration of OPG or both.[16,34]

On the basis of these findings, elevated reactivity for RANKL when compared to OPG would be expected in odontogenic cysts and tumours. Coherently, higher expression of RANKL compared to OPG has been demonstrated in solid ameloblastomas,[12] calcifying epithelial odontogenic tumours, odontogenic myxomas, and ameloblastic fibromas.[11] According to Andrade et al.[11] and da Silva et al.[12] these findings are consistent with the aggressive clinical behaviour of solid ameloblastomas and with the potential for recurrence and bone resorption of odontogenic myxomas and ameloblastic fibromas.

In the present study, analysis of the RANKL/OPG ratio in the epithelial lining and fibrous capsule revealed a RANKL = OPG ratio or RANKL < OPG ratio in most NSOKCs and SOKCs ($P > 0.05$), suggesting that differences in the biological behaviour of these lesions may not be related to this ratio. In agreement with these results, da Silva et al.[12] observed that sporadic OKCs tend to present RANKL = OPG ratios in the epithelial lining and RANKL < OPG ratios in the fibrous capsule. According to Andrade et al.[11] odontogenic lesions that are cystic in architecture but neoplastic in nature (e.g., unicystic ameloblastoma, and calcifying cystic odontogenic tumour) tend to present a lower RANKL/OPG ratio than solid tumours, a finding that might be related to the osteolytic potential of these lesions.

Nevertheless, in view of the potentially aggressive biological behaviour of OKCs,[3,32] the consistent expression of OPG in OKCs observed in the present study suggests the involvement of this protein in biological processes other than bone remodelling. In line with this suggestion, Sandra et al.[35] showed that treatment of an ameloblastoma-derived cell line with OPG significantly reduced the potential of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in these cells. These findings suggest that the expression of OPG in OKCs might be related to an antiapoptotic role of this protein in these lesions, particularly in the epithelial lining.

Studies have also demonstrated the involvement of RANKL and OPG in vascular biology, with these proteins being able to stimulate the survival, proliferation and migration of endothelial cells.[18–20] However, the present results showed no significant correlation between the angiogenic index and

expression of RANKL and OPG ($P > 0.05$), suggesting that these proteins may not be involved in the formation of new blood vessels in OKCs.

Angiogenesis is a complex process that is regulated by several intercellular pathways that exert both pro- and anti-angiogenic functions.[21,36] This process, which can be evaluated by determination of the angiogenic index, is critical for the development and progression of odontogenic cysts and tumours.[13,29,37] In addition, studies have shown a relationship between the angiogenic index and biological behaviour of odontogenic lesions.[13,37] In this respect, Alaeddini et al.[13] observed that microvessel density (MVD) is higher in solid ameloblastomas than in OKCs and dentigerous cysts, suggesting that angiogenesis is one of the mechanisms contributing to the different biological behaviours of these lesions. Similarly, Chen et al.[37] found a gradient in the vascularisation of OKCs, primary ameloblastomas, recurrent ameloblastomas, and malignant ameloblastomas, with lower MVD counts in OKCs and higher counts in malignant ameloblastomas. In the present study, no significant differences in the MVC were observed between NSOKCs and SOKCs ($P > 0.05$), indicating that differences in the biological behaviour of these lesions are not related to the angiogenic index.

Recently, Mitrou et al.[38] demonstrated a linear relationship between the expression of vascular endothelial growth factor (VEGF) and cell proliferation in the epithelial lining of OKCs. According to these authors, the expression of this proangiogenic factor by epithelial cells of OKCs might promote growth through an autocrine proliferative effect on the cystic epithelium. A similar proliferative effect has been demonstrated in biliary cysts developing in acquired dominant polycystic kidney disease.[39] Taken together, these findings and the present results regarding the angiogenic index of SOKCs and NSOKCs highlight the importance of further studies investigating the non-angiogenic functions of VEGF in OKCs.

Myofibroblasts are specialised contractile cells that have been implicated in a variety of diseases, such as fibrocontractive diseases and cancer, due to their ability to synthesise extracellular matrix components and to secrete proteases, cytokines and proangiogenic factors.[24–26] With respect to odontogenic lesions, Vered et al.[14] observed that the mean number of myofibroblasts was significantly higher in solid ameloblastomas and OKCs than in dentigerous cysts, unicystic ameloblastomas, and ameloblastic fibromas/fibroodontomas. According to these authors, myofibroblasts may contribute to the biological behaviour of aggressive odontogenic lesions. Recently, Fregnani et al.[15] showed that both the abundant presence of myofibroblasts and the expression of matrix metalloproteinase-2 (MMP-2) in solid ameloblastomas were significantly associated with rupture of cortical bone.

In the present study, myofibroblasts were detected in the fibrous capsule of primary NSOKCs, recurrent NSOKCs, and SOKCs. These findings agree with previous studies indicating these cells to be an important component of the fibrous capsule of OKCs.[14,40] However, analysis of the number of α-SMA-positive cells revealed no significant differences between groups ($P > 0.05$), suggesting that differences in the biological behaviour of SOKCs and NSOKCs may not be related to the number of myofibroblasts in these lesions.

Nevertheless, the possibility of quantitative and/or qualitative differences in the products synthesised by myofibroblasts in SOKCs and NSOKCs cannot be ruled out. This suggestion is supported by studies investigating the expression of MMPs. Cavalcante et al.[9] observed a higher expression of MMP-1 in the fibrous capsule of SOKCs when compared to NSOKCs. In addition, Leonardi et al.[10] showed strong immunoreactivity for MMP-13 in the fibrous capsule of SOKCs, whereas no expression of this protease was observed in NSOKCs. Studies have demonstrated that myofibroblasts constitute an important source of proteases such as MMPs-1, -2, -3, -9, and -13.[15,26,41,42] In this respect, the similar number of myofibroblasts in syndrome and non-syndrome OKCs observed in the present investigation suggests that the differences in the expression of MMP-1 and MMP-13 reported in the studies of Cavalcante et al.[9] and Leonardi et al.[10] may be partially related to differences in the synthesis capacity of myofibroblasts present in SOKCs and NSOKCs.

Myofibroblasts have been shown to play an important role in tumour angiogenesis. Konstantinopoulos et al.[43] demonstrated that myofibroblasts surrounding colon adenocarcinomas are an important source of VEGF and cyclooxygenase-2, two well-recognised proangiogenic factors. Using a gastric cancer mouse model, Guo et al.[44] observed that myofibroblasts stimulated by tumour cells express VEGF-A and other angiogenic factors, promoting angiogenesis. However, no significant correlation was found between the number of myofibroblasts and angiogenic index in the present study ($P > 0.05$). Therefore, myofibroblasts may not be primarily involved in the formation of new blood vessels in OKCs.

The present results suggest that differences in the biological behaviour of SOKCs and NSOKCs may not be related to the expression of RANKL and OPG, to the RANKL/OPG ratio, to the angiogenic index, or to the number of myofibroblasts in these lesions. In view of the lack of differences in the angiogenic index and in the number of myofibroblasts between these lesions, studies investigating possible qualitative differences in vascularisation and in the products synthesised by myofibroblasts, such as growth factors and proteases, may help explain the increased aggressiveness of OKCs related to Gorlin syndrome.

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Competing interest

None declared.

Ethical approval

The ethical approval for our research was given by the Ethics Committee of the Federal University of Rio Grande do Norte, Brazil (protocol number: 039/10).

REFERENCES

1. Philipsen HP. Keratocystic odontogenic tumor. In: Barnes L, Eveson JW, Reichart P, Sidransky D, editors. *World Health Organization classification of tumors: pathology and genetics of head and neck tumors*. Lyon: IARC Press; 2005.
2. Kichi E, Enokiya Y, Muramatsu T, Hashimoto S, Inoue T, Abiko Y, et al. Cell proliferation, apoptosis, and apoptosis-related factors in odontogenic keratocysts and dentigerous cysts. *J Oral Pathol Med* 2005;**34**:280–6.
3. Figueroa A, Correnti M, Avila M, Andea A, DeVilliers P, Rivera H. Keratocystic odontogenic tumor associated with nevoid basal cell carcinoma syndrome: similar behavior to sporadic type? *Otolaryngol Head Neck Surg* 2010;**142**:179–83.
4. Kimi K, Kumamoto H, Ooya K, Motegi K. Immunohistochemical analysis of cell-cycle- and apoptosis-related factors in lining epithelium of odontogenic keratocysts. *J Oral Pathol Med* 2001;**30**:434–42.
5. Manfredi M, Vescovi P, Bonanini M, Porter S. Nevoid basal cell carcinoma syndrome: a review of the literature. *Int J Oral Maxillofac Surg* 2004;**33**:117–24.
6. Díaz-Fernández JM, Infante-Cossío P, Belmonte-Caro R, Ruiz-Laza L, García-Perla-García A, Gutiérrez-Pérez JL. Basal cell nevus syndrome. Presentation of six cases and literature review. *Med Oral Patol Oral Cir Bucal* 2005;**10**: 57–66.
7. Kolář Z, Geierová M, Bouchal J, Pazdera J, Zboril V, Tvrdý P. Immunohistochemical analysis of the biological potential of odontogenic keratocysts. *J Oral Pathol Med* 2006;**35**:75–80.
8. Amorim RF, Godoy GP, Galvão HC, Souza LB, Freitas RA. Immunohistochemical assessment of extracellular matrix components in syndrome and non-syndrome odontogenic keratocysts. *Oral Dis* 2004;**10**:265–70.
9. Cavalcante RB, Pereira KM, Nonaka CF, Nogueira RL, de Souza LB. Immunohistochemical expression of MMPs 1, 7, and 26 in syndrome and nonsyndrome odontogenic keratocysts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;**106**:923–9.
10. Leonardi R, Matthews JB, Caltabiano R, Greco M, Lombardo C, Loreto C, et al. MMP-13 expression in keratocyst odontogenic tumor associated with NBCCS and sporadic keratocysts. *Oral Dis* 2010;**16**:795–800.
11. Andrade FR, Sousa DP, Mendonça EF, Silva TA, Lara VS, Batista AC. Expression of bone resorption regulators (RANK, RANKL, and OPG) in odontogenic tumors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;**106**:548–55.
12. da Silva TA, Batista AC, Mendonça EF, Leles CR, Fukada S, Cunha FQ. 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**:333–41.
13. Alaeddini M, Salah S, Dehghan F, Eshghyar N, Etemad-Moghadam S. Comparison of angiogenesis in keratocystic odontogenic tumors, dentigerous cysts and ameloblastomas. *Oral Dis* 2009;**15**:422–7.
14. Vered M, Shohat I, Buchner A, Dayan D. Myofibroblasts in stroma of odontogenic cysts and tumors can contribute to variations in the biologic behavior of lesions. *Oral Oncol* 2005;**41**:1028–33.
15. Fregnani ER, Sobral LM, Alves FA, Soares FA, Kowalski LP, Coletta RD. Presence of myofibroblasts and expression of matrix metalloproteinase-2 (MMP-2) in ameloblastomas correlate with rupture of the osseous cortical. *Pathol Oncol Res* 2009;**15**:231–40.
16. Boyce BF, Xing L. Functions of RANK/RANKL/OPG in bone modeling and remodeling. *Arch Biochem Biophys* 2008;**473**:139–46.
17. Kearns AE, Khosla S, Kostenuik PJ. Receptor activator of nuclear factor κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 2008;**29**:155–92.
18. Baud'huin M, Lamoureux F, Duplomb L, Rédini F, Heymann D. RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases. *Cell Mol Life Sci* 2007;**64**:2334–50.
19. Kobayashi-Sakamoto M, Isogai E, Hirose K, Chiba I. Role of alphav integrin in osteoprotegerin-induced endothelial cell migration and proliferation. *Microvasc Res* 2008;**76**:139–44.
20. McGonigle JS, Giachelli CM, Scatena M. Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function. *Angiogenesis* 2009;**12**:35–46.
21. Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. *J Burn Care Res* 2010;**31**:158–75.
22. Clapp C, Thebault S, Jeziorski MC, Martínez De La Escalera G. Peptide hormone regulation of angiogenesis. *Physiol Rev* 2009;**89**:1177–215.
23. Nikitenko LL. Vascular endothelium in cancer. *Cell Tissue Res* 2009;**335**:223–40.
24. Gabbiani G. The myofibroblasts in wound healing and fibrocontractive diseases. *J Pathol* 2003;**200**:500–3.
25. Eyden B, Banerjee SS, Shenjere P, Fisher C. The myofibroblast and its tumors. *J Clin Pathol* 2009;**62**:236–49.
26. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 2008;**123**:2229–38.
27. Kramer IRH, Pindborg JJ, Shear M. *Histological typing of odontogenic tumors*. 2nd ed. Berlin: Springer; 1992.
28. Evans DG, Ladusans EJ, Rimmer S, Burnell LD, Thakker N, Farndon PA. Complications of the naevoid basal cell carcinoma syndrome: results of a population based study. *J Med Genet* 1993;**30**:460–4.
29. Nonaka CF, Maia AP, Nascimento GJ, de Almeida Freitas R, Batista de Souza L, Galvão HC. Immunoexpression of vascular endothelial growth factor in periapical granulomas, radicular cysts, and residual radicular cysts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;**106**:896–902.
30. Maeda K, Chung YS, Takatsuka S, Ogawa Y, Onoda N, Sawada T, et al. Tumour angiogenesis and tumour cell proliferation as prognostic indicators in gastric carcinoma. *Br J Cancer* 1995;**72**:319–23.
31. Sun LS, Li XF, Li TJ. PTCH1 and SMO gene alterations in keratocystic odontogenic tumors. *J Dent Res* 2008;**87**:575–9.
32. Hakim SG, Kosmehl H, Sieg P, Trenkle T, Jacobsen HC, Attila Benedek G, et al. Altered expression of cell-cell adhesion molecules β-catenin/E-cadherin and altered Wnt-signaling pathway in sporadic and syndromal keratocystic odontogenic tumors. *Clin Oral Invest* 2011;**15**:321–8.
33. Anandarajah AP. Role of RANKL in bone diseases. *Trends Endocrinol Metab* 2009;**20**:88–94.
34. Cochran DL. Inflammation and bone loss in periodontal disease. *J Periodontol* 2008;**79**:1569–76.
35. Sandra F, Hendarmin L, Nakamura S. Osteoprotegerin binds with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): suppression of TRAIL-induced apoptosis in ameloblastomas. *Oral Oncol* 2006;**42**:415–20.
36. Gordon MS, Mendelson DS, Kato G. Tumor angiogenesis and novel antiangiogenic strategies. *Int J Cancer* 2010;**126**: 1777–87.
37. Chen WL, Ouyang KX, Li HG, Huang ZQ, Li JS, Wang JG. Expression of inducible nitric oxide synthase and vascular endothelial growth factor in ameloblastoma. *J Craniofac Surg* 2009;**20**:171–5.
38. Mitrou GK, Tosios KI, Kyroudi A, Sklavounou A. Odontogenic keratocyst expresses vascular endothelial growth factor: an immunohistochemical study. *J Oral Pathol Med* 2009;**38**:470–5.

39. Fabris L, Cadamuro M, Fiorotto R, Roskams T, Spirlì C, Melero S, et al. Effects of angiogenic factor overexpression by human and rodent cholangiocytes in polycystic liver diseases. *Hepathology* 2006;**43**:1001–12.
40. Lombardi T, Morgan PR. Immunohistochemical characterisation of odontogenic cysts with mesenchymal and myofilament markers. *J Oral Pathol Med* 1995;**24**:170–6.
41. Lederle W, Hartenstein B, Meides A, Kunzelmann H, Werb Z, Angel P, et al. MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. *Carcinogenesis* 2010;**31**:1175–84.
42. McKaig BC, McWilliams D, Watson SA, Mashida YR. Expression and regulation of tissue inhibitor of matrix metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* 2003;**162**:1355–60.
43. Konstantinopoulos PA, Vandoros GP, Karamouzis MV, Gkermpesi M, Sotiropoulou-Bonikou G, Papavassiliou AG. EGF-R is expressed and AP-1 and NF-κB are activated in stromal myofibroblasts surrounding colon adenocarcinomas paralleling expression of COX-2 and VEGF. *Cell Oncol* 2007;**29**:477–82.
44. Guo X, Oshima H, Kitmura T, Taketo MM, Oshima M. Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer. *J Biol Chem* 2008;**283**:19864–71.