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

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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.

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

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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