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Cell proliferation and apoptosis in keratocystic odontogenic tumors

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Abstract

Objectives: Keratocystic odontogenic tumors (KOTs), also known as odontogenic keratocysts, were recently classified as a benign neoplasia due to the aggressive clinical behavior. Although several studies have shown the high proliferative activity of the epithelial lining, few studies have evaluated apoptosis in KOTs. Therefore, the aim of this study is to evaluate and compare the proliferation index (PI) and the apoptotic index (AI) of the epithelial lining in sporadic KOTs, KOTs associated with the Nevoid Basal Cell Carcinoma Syndrome (NBCCS KOTs), and dentigerous cysts.

Material and methods: A total of 11 sporadic KOTs, 15 NBCCS KOTs, and 11 dentigerous cysts were evaluated. The PI was assessed by immunohistochemical detection of the cell proliferation marker Ki-67. The AI was assessed by morphological evaluation of sections stained by methyl green-pyronin. The TUNEL assay was used to confirm the occurrence of apoptosis. Differences in the PI and the AI between sporadic KOTs, NBCCS KOTs, and dentigerous cysts were analyzed using the Kruskal-Wallis test. Differences in the PI and the AI between the epithelial layers of each lesion were analyzed using the Wilcoxon test.

Results: The PI and AI were higher in sporadic and NBCCS KOTs than in dentigerous cysts. No difference in these indexes was observed between sporadic and NBCCS KOTs. In dentigerous cysts, the PI was higher in the basal layer. In sporadic and NBCCS KOTs, the PI was higher in suprabasal layer. No difference in the AI was observed between the basal layer and the suprabasal layer in the three lesions. The AI was higher in the superficial layer of sporadic and NBCCS KOTs.

Conclusions: The present study demonstrates that the epithelial lining of KOTs shows a distinct pattern of cell proliferation and apoptosis, reflecting its high cell turnover and reinforcing its classification as an odontogenic tumor.

Key words: *Keratocyst, keratocystic odontogenic tumor, nevoid basal cell carcinoma syndrome, dentigerous cyst, apoptosis, mitosis.*

Introduction

Since its first description in 1956 (1), the odontogenic keratocyst has been deeply studied due to its aggressive clinical behavior with high recurrence rates (2,3), distinct histopathologic features (4), singular growth mechanism (5,6), and genetic alterations (7,8). It may also be associated with the Nevoid Basal Cell Carcinoma Syndrome (NBCCS), an autosomal-dominant disease characterized by several developmental abnormalities and a predisposition to neoplasm development (9). The potential for aggressive clinical behavior and local recurrence resulted in its recent classification as a benign odontogenic tumor with a new nomenclature: keratocystic odontogenic tumor (10).

Apoptosis is a type of cell death characterized by an organized series of biochemical and morphological events. It is an essential mechanism in development and adult tissue homeostasis, controlling cell turnover in normal and neoplastic tissues in cooperation with cell proliferation (11-13). Several studies have demonstrated the higher proliferation activity of the epithelial lining in keratocystic odontogenic tumors (KOTs) in relation to odontogenic cysts (14-19). Moreover, some authors have also demonstrated that this proliferation is higher in KOTs associated with the NBCCS (14,16,20). However, despite the fact that cell turnover is controlled by both cell proliferation and apoptosis, few studies have evaluated apoptosis related proteins (16,21-23) and apoptotic index (17,19) in the epithelial lining of the odontogenic cysts.

Therefore, the aim of this study is to evaluate and compare the proliferation index (PI) and the apoptotic index (AI) of the epithelial lining in KOTs not associated with the NBCCS (sporadic KOTs), KOTs associated with the NBCCS (NBCCS KOTs), and dentigerous cysts.

Material and Methods

- *Tissues and samples*

This study was approved by the local ethics committee. A total of 11 sporadic KOTs, 15 NBCCS KOTs, and 11 dentigerous cysts from archival formalin-fixed, paraffin-embedded specimens were evaluated. The 15 NBCCS KOTs were from patients fulfilling the diagnostic criteria for NBCCS (9) and the 11 sporadic KOTs were from patients with neither signs nor symptoms of NBCCS.

- *Assessment of the proliferation index (PI)*

The PI was assessed by immunohistochemical detection of the cell proliferation marker Ki-67. Four μm sections from the paraffin-embedded samples were used. Tissue sections were dewaxed with xylene, hydrated using graded alcohols, and treated with 0.6% H_2O_2 in methanol to eliminate endogenous peroxidase activity. Antigen retrieval was conducted by heating in a 0.01 M citrate buffer (pH 6.0) for 30 minutes. Subsequently, the anti-Ki-67 monoclonal antibody was used (clone MM1, diluted 1:100; Novocastra Laboratories, Newcastle, UK). The LSAB+ kit (Dako Corporation, Carpinteria, USA) was used for application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer's instructions. The reactive products were visualized by immersing the sections for 3 min in 0.03% diaminobenzidine solution, containing 2 mM H_2O_2. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted. Sections of oral squamous cell carcinoma with known Ki-67 immunoreactivity were used as a positive control. Negative control was determined by omission of the primary antibody. Cell counts were made at x400 magnification, using an eyepiece grid in light microscopy for at least 10 fields. Epithelial cells with distinct brown nuclear staining were regarded as Ki-67 positive (Fig. 1). The percentage of positive cells was then calculated to obtain the PI.

- *Assessment of the apoptotic index (AI)*

The AI was assessed by morphological evaluation of sections stained by methyl green-pyronin. Four μm sections from the paraffin-embedded samples were used. Tissue sections were dewaxed with xylene and hydrated using graded alcohols. The sections were stained for 5 minutes in a solution prepared with 70 mL of 2% methyl green and 30 mL of 1% pyronine. Subsequently, the sections were enveloped in a paper filter for 5 minutes and washed individually in distilled water. The sections were then evaluated under light microscopy and, if the specimens appeared as exceedingly red, they were washed again with 80% ethanol at 5°C. The sections were then dehydrated and mounted. Cell counts were made at x1000 magnification, using an eyepiece grid under light microscopy for at least 10 fields. Epithelial cells with morphologic characteristics of apoptosis were regarded as apoptotic cells (Fig. 2). The percentage of apoptotic cells was then calculated to obtain the AI.

The TUNEL assay was used qualitatively, to confirm the occurrence of apoptosis in the epithelial lining of the lesions (Fig. 3). The commercially available TdT-FragEL™ DNA Fragmentation Detection kit (Calbiochem, Oncogene Research Products, Cambridge, USA) was used according to the manufacturer's instructions. Briefly, four μm sections from the paraffin-embedded samples were dewaxed with xylene and hydrated using graded alcohols. Afterward, the specimens were treated with 20 mg/mL proteinase K for 5 minutes and with 0.6% H2O2 in methanol to eliminate endogenous peroxidase activity. Subsequently, the sections were treated with TDT enzyme and immersed in a biotinylated nucleotides solution. Labeled cells were detected using streptavidin-peroxidase conjugate followed by diaminobenzidine staining.

- *Statistical analysis*

The data were analyzed by means of BioEstat 3.0 software (Optical Digital Technology, Belém, Brazil). Differences in the PI and the AI among sporadic KOTs, NBCCS KOTs, and dentigerous cysts were analyzed using the Kruskal-Wallis test. Differences in the PI and the AI between the

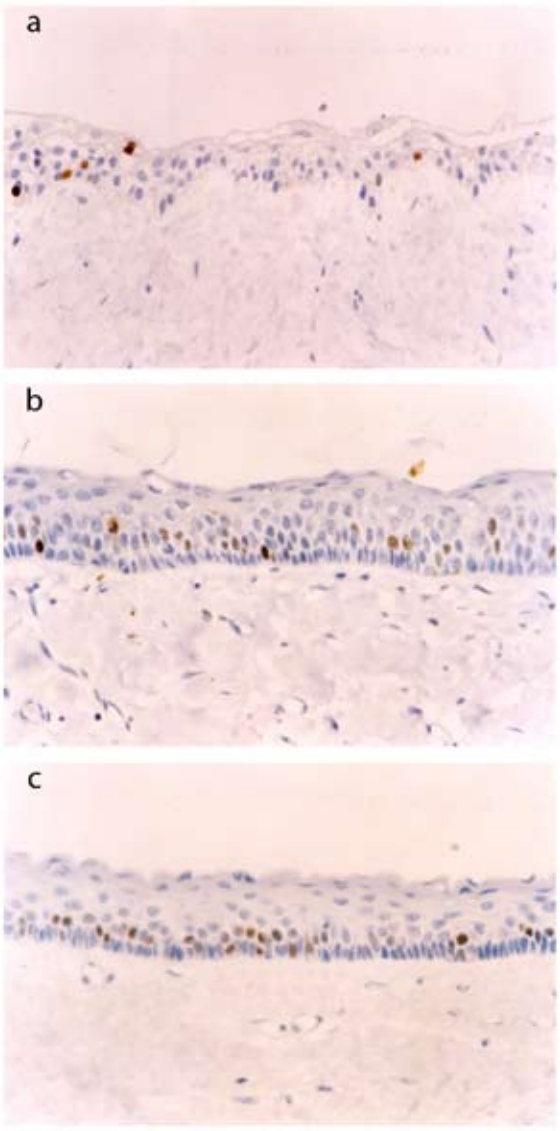

Fig. 1 Immunohistochemical reactivity for Ki-67 in dentigerous cysts (a), sporadic KOTs (b), and NBCCS KOTs (c). Cells with distinct nuclear staining were regarded as Ki-67 positive (original magnification: x400).

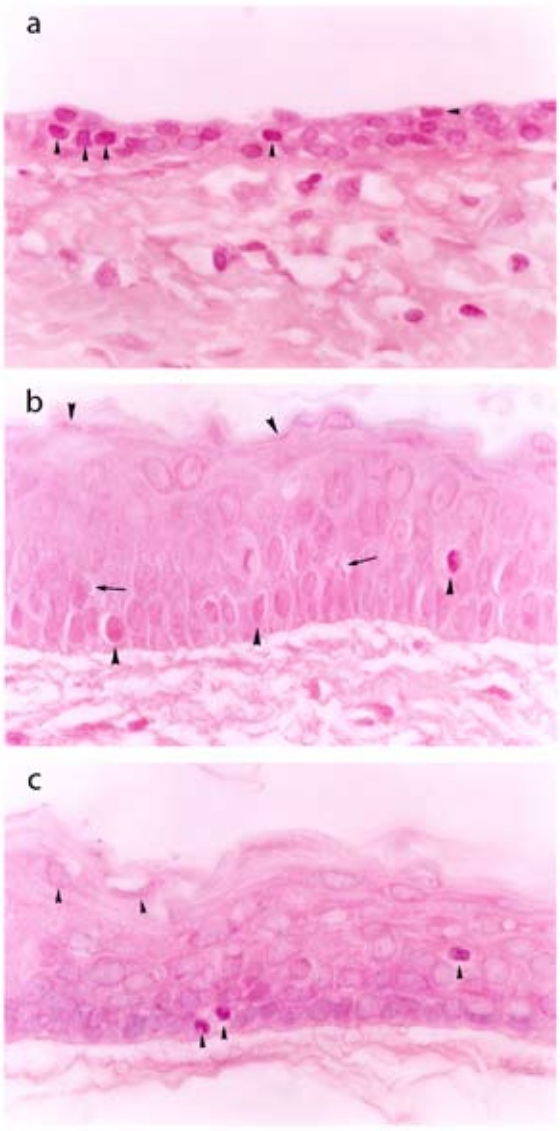

Fig. 2. Methyl green-pyronine staining of dentigerous cysts (a), sporadic KOTs (b), and NBCCS KOTs (c). Apoptotic cells (arrowheads) and apoptotic bodies (arrows) can be visualized in the epithelial lining (original magnification: x1000).

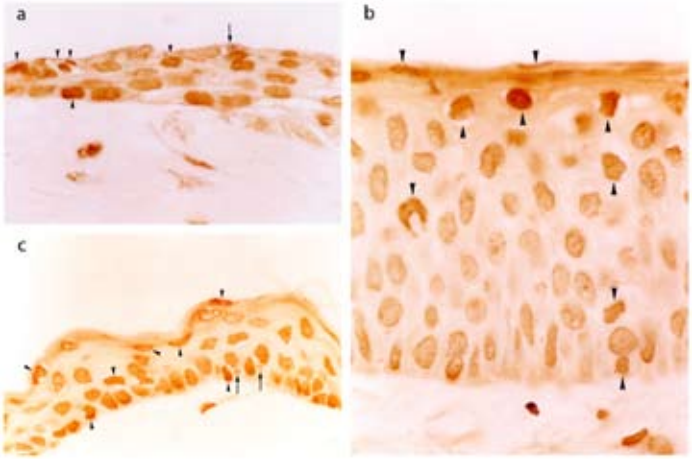

Fig. 3. TUNEL labeling of dentigerous cysts (a), sporadic KOTs (b), and NBCCS KOTs (c). Apoptotic cells (arrowheads) and apoptotic bodies (arrows) can be visualized in the epithelial lining (original magnification: x1000).

Table 1. Median value and range of proliferation index (PI) and apoptotic index (AI) of the epithelial lining in dentigerous cysts, sporadic KOTs, and NBCCS KOTs.

		Dentigerous cysts	Sporadic KOTs	NBCCS KOTs	P-value [1]
PI	Median	2.90	9.83	8.18	<0.05 [a]
	Range	1.73-7.49	7.19-19.78	5.99-14.83	<0.05 [b] n.s. [c]
AI	Median	12.24	18.35	18.30	<0.05 [a]
	Range	8.75- 14.98	13.23-29.99	7.69-27.83	<0.05 [b] n.s. [c]

1 P-values were obtained through the Kruskal-Wallis test
a dentigerous cysts vs. sporadic KOTs
b dentigerous cysts vs. NBCCS KOTs
c sporadic KOTs vs. NBCCS KOTs
n.s., not significant

Table 2. Median value and range of proliferation index (PI) and apoptotic index (AI) of the epithelial layers in dentigerous cysts, sporadic KOTs, and NBCCS KOTs.

			Basal	Suprabasal	Superficial	P-value [1]
PI	Dentigerous cysts	Median	4.57	2.00	-	<0.05 [a]
		Range	1.76-12.18	0.73-6.78	-	
	Sporadic KOTs	Median	3.52	16.57	0	<0.05 [a]
		Range	0.92-27.25	10.93-34.09	0	<0.05 [b] <0.05 [c]
	NBCCS KOTs	Median	4.62	14.03	0	<0.05 [a]
		Range	0.78-12.09	8.23-22.20	0	<0.05 [b] <0.05 [c]
	P-value [2]		n.s. [d] n.s. [e] n.s. [f]	<0.05 [d] <0.05 [e] n.s. [f]	n.s. [f]	
AI	Dentigerous cysts	Median	11.11	12.67	-	ns. [a]
		Range	6.36-19.32	8.44-16.67	-	
	Sporadic KOTs	Median	9.41	10.25	99.31	ns. [a]
		Range	4.96-10.98	6.78-17.62	79.37-100	<0.05 [b] <0.05 [c]
	NBCCS KOTs	Median	10.48	10.89	95.65	ns. [a]
		Range	5.70-13.56	3.93-21.00	71.43-100	<0.05 [b] <0.05 [c]
	P-value [2]		n.s. [d] n.s. [e] n.s. [f]	n.s. [d] n.s. [e] n.s. [f]	n.s. [f]	

1 P-values were obtained through the Wilcoxon test
a Basal vs. Suprabasal
b Basal vs. Superficial
c Suprabasal vs. Superficial

2 P-values were obtained through the Kruskal-Wallis test
d dentigerous cysts vs. sporadic KOTs
e dentigerous cysts vs. NBCCS KOTs
f sporadic KOTs vs. NBCCS KOTs
n.s., not significant

epithelial layers of each lesion were analyzed using the Wilcoxon test. Tests were considered significant when their P-values were < 0.05.

Results

The PI and the AI were higher in sporadic KOTs and NBCCS KOTs than in dentigerous cysts ($p<0.05$). No difference in these indexes was observed between sporadic KOTs and NBCCS KOTs ($p>0.05$) (Table 1).

In dentigerous cysts, the PI was higher in the basal layer than in the suprabasal layer ($p<0.05$). In sporadic KOTs and NBCCS KOTs, the PI was higher in the suprabasal layer than in the basal layer ($p<0.05$). Although there were no differences in the PI of the basal layer among the three lesions ($p>0.05$), the PI of the suprabasal layer was higher in sporadic KOTs and NBCCS KOTs than in dentigerous cysts ($p<0.05$) (Table 2).

No differences were observed in the AI between the basal layer and the suprabasal layer in the three lesions ($p>0.05$). In sporadic KOTs and NBCCS KOTs, the AI was higher in the superficial layer ($p<0.05$). There were no differences in the AI of the basal layer and in the AI of the suprabasal layer among the three lesions ($p>0.05$). There were also no differences in the AI of the superficial layer when comparing sporadic KOTs with NBCCS KOTs ($p>0.05$) (Table 2).

Discussion

Although cell proliferation of the epithelial lining has been exhaustively studied in odontogenic cysts (14-20,22,23), few studies have assessed apoptosis in these lesions (16,17,19,21-23) despite the fact that cell turnover is controlled by cell proliferation in cooperation with apoptosis (12). The main aim of the present study was to evaluate cell proliferation and apoptosis in sporadic KOTs and NBCCS KOTs, lesions with aggressive clinical behavior, and a growth mechanism related to the proliferative activity of the epithelial lining (14,15,17-19). Dentigerous cysts were used for comparison due to its less aggressive clinical behavior and a growth mechanism related to fluid accumulation (5,24).

The PI was assessed by immunohistochemical detection of Ki-67, one of the most used and trustworthy proliferation markers (25). The AI was assessed by the morphological evaluation of sections stained by the methyl green-pyronine. This histochemical staining method provides good morphological detection of apoptotic cells (26). The TUNEL assay was used qualitatively, to confirm the occurrence of apoptosis in the epithelial lining of the lesions evaluated.

The PI was higher in sporadic KOTs and NBCCS KOTs than in dentigerous cysts (Table 1). This is in agreement with previous reports (14,15,17-19) and may well be related to the distinct growth mechanism of these lesions. As dentigerous cysts grow by means of fluid accumulation (5,24), cell proliferation of its epithelial lining occurs secondarily to cyst expansion. In contrast, the high proliferative activity of the KOTs epithelial lining is pinpointed as its main growth mechanism (8,14,17-19), which supports its classification as an odontogenic tumor (8,10). A recent study has reported that KOTs, but not dentigerous cysts, show intraepithelial deposition of perlecan, a heparan sulfate proteoglycan present in several neoplasms and pointed as a requirement for cell proliferation (27). This high proliferative activity could be a consequence of genetic alterations demonstrated in sporadic and NBCCS KOTs (7,8).

In dentigerous cysts, the PI was higher in the basal layer than in the suprabasal layer (Table 2). In sporadic and NBCCS KOTs, the PI was higher in the suprabasal layer (Table 2). Similar results have been previously reported (14-20,22), showing that the proliferative center of the KOTs epithelial lining is the suprabasal layer and reinforcing the fact that this epithelial lining presents a different pattern of cell proliferation and differentiation.

The AI was higher in sporadic and NBCCS KOTs than in dentigerous cysts (Table 1). Similar results from previous studies have been observed with respect to dentigerous cysts and sporadic KOTs (17,19). It is important to emphasize that the high PI and AI in the epithelial lining in sporadic and NBCCS KOTs demonstrate its high cell turnover and reinforce its classification as an odontogenic tumor.

Although the present study detected apoptotic cells in all epithelial layers of the three lesions (Table 2), other studies have observed apoptotic cells only in the suprabasal layer of dentigerous cysts as well as in the superficial layer of sporadic KOTs (17,19). This divergence seems to be a consequence of methodological difficulties of apoptosis detection assays, which can be responsible for differing results observed in analogous studies (28). Even though previous studies have shown immunoexpression of anti-apoptotic protein bcl-2 limited to the basal layer of sporadic and NBCCS KOTs (19,21,22), no difference was observed in the AI among basal and suprabasal layers in these lesions (Table 2). This fact could be explained by the complexity of apoptosis regulation and the large numbers of molecular players involved in the apoptotic signaling pathways (11-13). In sporadic KOTs and NBCCS KOTs, the AI was higher in the superficial layer, which should explain why these lesions, despite their high proliferative activity, are observed as cystic lesions but not as tumor masses (19).

No differences in the PI and the AI were observed between sporadic and NBCCS KOTs (Table 1). There were also no differences in the PI and the AI of the epithelial layers when comparing sporadic KOTs with NBCCS KOTs (Table 2). Although similar results related to cell proliferation had in fact been observed (22), a higher PI (14,16,20), and a higher AI (16) were demonstrated in

NBCCS KOTs. These data suggest that the more aggressive clinical behavior of NBCCS KOTs in relation to sporadic KOTs is not related to differences in the cell turnover of the epithelial lining, but to the multiplicity of lesions and early development of KOTs in NBCCS (29).

In conclusion, the present study demonstrates that the epithelial lining of KOTs shows a distinct pattern of cell proliferation and apoptosis, reflecting its high cell turnover and reinforcing its classification as an odontogenic tumor.

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