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Immunohistochemical study of the plasminogen activator system in benign epithelial odontogenic lesions

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Abstract: The aim of this study was to analyze and compare the immunohistochemical expression of plasminogen activator system (PAS) proteins (uPA, uPAR, and PAI-1) in ameloblastomas (AMBs), odontogenic keratocysts (OKCs), and dental follicles (DFs) representing normal odontogenic tissue, as well as to investigate possible correlations between these proteins. Twenty AMBs, 20 OKCs, and 10 DFs were selected for immunohistochemical analysis. In each case, the immunoexpression of uPA, uPAR, and PAI-1 was evaluated semiquantitatively based on the percentage of positivity in odontogenic epithelial and connective tissue cells. The epithelial immunoexpression of uPA was significantly lower in AMBs when compared to OKCs ($p = 0.001$) and DFs ($p = 0.029$). Significantly higher epithelial immunostaining for uPAR was observed in AMBs when compared to OKCs ($p < 0.001$). There were no significant differences in the epithelial immunoexpression of PAI-1 between AMBs and OKCs ($p = 1.000$). The correlations found for the expression of the studied proteins were not statistically significant ($p > 0.05$). However, the epithelial and connective tissue expressions of uPAR have a strong positive and statistically significant correlation in AMBs. The present results suggest that uPA is involved in the pathogenesis of OKCs and that uPAR may participate in tumorigenesis in AMBs. The high percentage of PAI-1-positive cells suggests a possible role for this protein in the development of AMBs and OKCs. Furthermore, the studied proteins do not seem to act synergistically in AMBs, OKCs, and DFs.

Keywords: Urokinase-type Plasminogen Activator; Plasminogen Inactivators; Odontogenic Tumors; Odontogenic Cysts; Immunohistochemistry.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Odontogenic cysts and tumors arise from tooth-forming tissues and exhibit different biological behaviors. Among these lesions, ameloblastoma (AMB) and odontogenic keratocyst (OKC) are particularly important because of their aggressive biological behavior and high recurrence rate.[1,2] Within this context, studies have investigated the pathogenesis of these lesions in order to develop new therapeutic strategies.[2,3]

The plasminogen activator system (PAS) is involved in the regulation of extracellular matrix (ECM) remodeling through the conversion of plasminogen to plasmin. In addition to its proteolytic activity, plasmin indirectly activates matrix metalloproteinases (MMPs), which are capable of cleaving ECM proteins. Urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR), and plasminogen activator inhibitor 1 (PAI-1) are the main components of the PAS.[4-9] This system has been extensively studied in malignant neoplasms. Several studies indicate that, in addition to causing ECM proteolysis, PAS proteins perform different roles in tumorigenesis, such as proliferation, apoptosis, local invasion, angiogenesis, and metastasis.[4,5,8,10] Studies indicate that uPA and PAI-1 are associated with prognosis in many types of cancer, such as breast, lung, stomach, and ovarian cancers. uPA can be used as a potential biomarker of aggressiveness and PAI-1 is related to poor prognosis and increased risk of metastasis.[10] Nevertheless, few studies have evaluated the role of the PAS in odontogenic lesions.[11-13]

In view of the aggressive behavior and high recurrence of AMBs and OKCs, the present study evaluated the immunoexpression levels of uPA, uPAR, and PAI-1 in AMBs and OKCs and compared them with the immunoexpression in dental follicles (DFs) in order to achieve a better understanding of the role of these proteins in the development of these lesions.

Methodology

This retrospective study was approved by the Research Ethics Committee of the Federal University of Rio Grande do Norte, Natal, Brazil (Process no. 3.447.864). Fifty tissue specimens including 20 AMBs, 20 OKCs, and 10 DFs, obtained from the archives of the Division of Oral Pathology, were selected for this study. Only cases with a sparse or absent inflammatory infiltrate and OKCs of patients without Gorlin syndrome were included. The morphological characteristics of the specimens were examined in hematoxylin/eosin-stained histological sections (5 µm thick) under light microscopy, in accordance to the 2017 WHO Classification.[14]

Immunohistochemistry

Histological sections (3 µm) were obtained from the paraffin-embedded specimens and mounted on glass slides with organosilane adhesive (3-aminopropyltriethoxysilane; Sigma Chemical Co., St. Louis, USA). For deparaffinization, rehydration, and antigen retrieval, the sections were immersed in Trilogy solution (1:100; Cell Marque, Rocklin, USA) in a Pascal pressure cooker. After these steps, endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide. The sections were incubated overnight in a moist chamber with the following primary antibodies: anti-uPA (sc-14019, 1:800, Santa Cruz Biotechnology, Dallas, USA), anti-uPAR (E-3, 1:200, Santa Cruz Biotechnology, Dallas, USA), and anti-PAI-1 (C-9, 1:400, Santa Cruz Biotechnology, Dallas, USA). The sections were then rinsed twice in phosphate-buffered saline (PBS) and incubated with the HiDef Detection HRP Polymer System (Cell Marque, Rocklin, USA) at room temperature. The reactions were developed using diaminobenzidine (Liquid DAB + Substrate; Dako, Carpinteria, USA) as chromogen. Finally, the sections were counterstained with Mayer's hematoxylin and coverslipped. Endothelial cells present in the histological sections were used as positive internal control. Sections in which the primary antibodies were omitted served as negative control.

Immunohistochemical assessment and statistical analysis

After immunohistochemical processing, as previously described by Khot et al.,[15] photomicrographs were obtained from five representative fields (×400) of odontogenic epithelial and connective tissue cells of each case using a camera coupled to the light microscope (Olympus CX31, Tokyo, Japan). The fields were evaluated by a single examiner on two different occasions. The examiner had been previously trained by an experienced oral pathologist. The kappa (k) coefficient was determined to confirm intraobserver reproducibility, which ranged from 0.846 to 1.0, indicating very good reproducibility.[16] Immunoexpression of uPA, uPAR, and PAI-1 was analyzed in odontogenic epithelial cells and fibroblasts

from connective tissue, and those exhibiting brown staining in the membrane and/or cytoplasm were classified as immunopositive. The percentage of positive cells was analyzed semiquantitatively in each field using an adaptation of the method proposed by Magnussen et al.[17] The following immunostaining scores for both cell types were established: 0 (0%), 1 (≤ 10%), 2 (11–50%), 3 (51–80%), and 4 (> 80%). The staining intensity was evaluated for descriptive purposes and classified as follows: 0 (absent), 1 (weak), 2 (moderate), or 3 (strong).[17] The median immunostaining score and median staining intensity score were determined for each case.

The data were analyzed statistically using the IBM SPSS Statistics program (version 25.0; IBM Corp., Armonk, USA). The nonparametric Kruskal-Wallis (*KW*) and Mann-Whitney (*U*) tests were applied, in addition to Spearman's correlation test (r). The level of significance was set at 5% ($p < 0.05$) for all statistical tests.

Results

Morphological features

The follicular and plexiform histopathological patterns were the most common subtypes of AMBs, which were detected in all cases. Squamous metaplasia and cystic degeneration were observed in 11 (55%) and 14 (70%) cases, respectively. There was only one case (5%) with follicles and central cells exhibiting granular differentiation. The OKC cases showed the typical features of the lesion, including a parakeratinized stratified squamous epithelium with corrugated surface, and basal cells with hyperchromatic nuclei in a palisade arrangement. The capsule was composed of fibrous connective tissue with areas of epithelial detachment. In DFs, discontinuous areas of simple epithelium compatible with reduced epithelium of the enamel organ were observed, in addition to epithelial nests and cords amidst connective tissue.

Epithelial expression of uPA

Immunoexpression of uPA in odontogenic epithelium was mainly cytoplasmic and diffuse. In AMBs, no differences were found in immunostaining characteristics between the central and peripheral regions of follicles or cords of tumor cells (Figure 1A). However, staining was greater in areas of squamous metaplasia than in adjacent cells. The staining intensity in cells with granular differentiation was similar to that of surrounding cells. Most OKCs exhibited uniform staining in the epithelium (Figure 1B). More intense staining, however, was observed in the parakeratin layer in 10% of cases. In DF cases, the immunostaining pattern was also diffuse both in the reduced enamel epithelium (Figure 1C) and in epithelial nests. Positive immunostaining for uPA was observed in 95% of AMBs, 100% of OKCs, and 90% of DFs (Table 1). The staining intensity was predominantly weak in AMBs and DFs (75% and 50% of cases, respectively), while most OKCs exhibited moderate staining (55%) (Table 1).

There was a significant difference ($p = 0.002$) in the immunoexpression of uPA between the groups analyzed (Table 2). Higher uPA expression was observed in DFs ($p = 0.029$) and OKCs ($p = 0.001$) when compared to AMBs (Table 3).

Epithelial expression of uPAR

Membrane and cytoplasmic immunoexpression of uPAR was generally observed. Most AMB cases (90%) showed immunopositivity for uPAR, with 70% classified as score 1 (Figure 1D) (Table 1). In some cases, staining mainly occurred in areas of squamous metaplasia, cystic degeneration, and granular differentiation. Most OKCs (75%) and DFs (60%) were negative for this protein (Figure 1E; 1F) (Table 1). Conversely, in positive OKCs, immunoexpression was focal and indistinct, while membrane staining in the reduced enamel epithelium was observed in some DFs. Regarding the staining intensity of uPAR-immunopositive cases, there was a higher frequency of weak intensity in all groups analyzed (Table 1).

Comparison of uPAR immunopositivity between AMBs, OKCs, and DFs revealed a statistically significant difference ($p = 0.001$) (Table 2). Immunoexpression of uPAR was significantly higher in AMBs than in OKCs ($p < 0.001$). AMBs did not differ significantly from DFs ($p = 0.302$), nor did OKCs from DFs ($p = 0.173$) in terms of uPAR immunoexpression (Table 3).

Epithelial expression of PAI-1

Diffuse and indistinct membrane and cytoplasmic immunoexpression of PAI-1 was observed in odontogenic epithelial cells of the studied groups. AMBs exhibited a similar immunostaining pattern in peripheral and central tumor cells (Figure 1G) and in areas of squamous metaplasia and granular differentiation. Most OKCs also showed an indistinct staining pattern (Figure 1H). However, a lower intensity or absence of staining was found in the parakeratin layer of some cases. In DFs, the immunostaining pattern was diffuse and there were no differences between the reduced enamel epithelium and odontogenic epithelial nests/cords (Figure 1I).

All AMBs, OKCs, and DFs were immunopositive for PAI-1. Score 4 was observed in 95% of AMBs and OKCs and in 100% of DFs (Table 1). In all groups, the staining intensity was moderate in most cases (Table 1). No variation in PAI-1 immunopositivity scores was observed in DFs. Thus, the Mann-Whitney (*U*) test was applied to compare PAI-1 immunopositivity scores between AMBs and OKCs. The results of this test did not show statistically significant differences between AMBs and OKCs (p = 1.000).

Connective tissue expression of uPA, uPAR, and PAI-1

The distribution of AMBs, OKCs, and DFs according to the scores of immunopositivity and intensity for uPA, uPAR, and PAI-1 are described in Table 4. A score of 2 was obtained in 50% of AMBs, 50% of

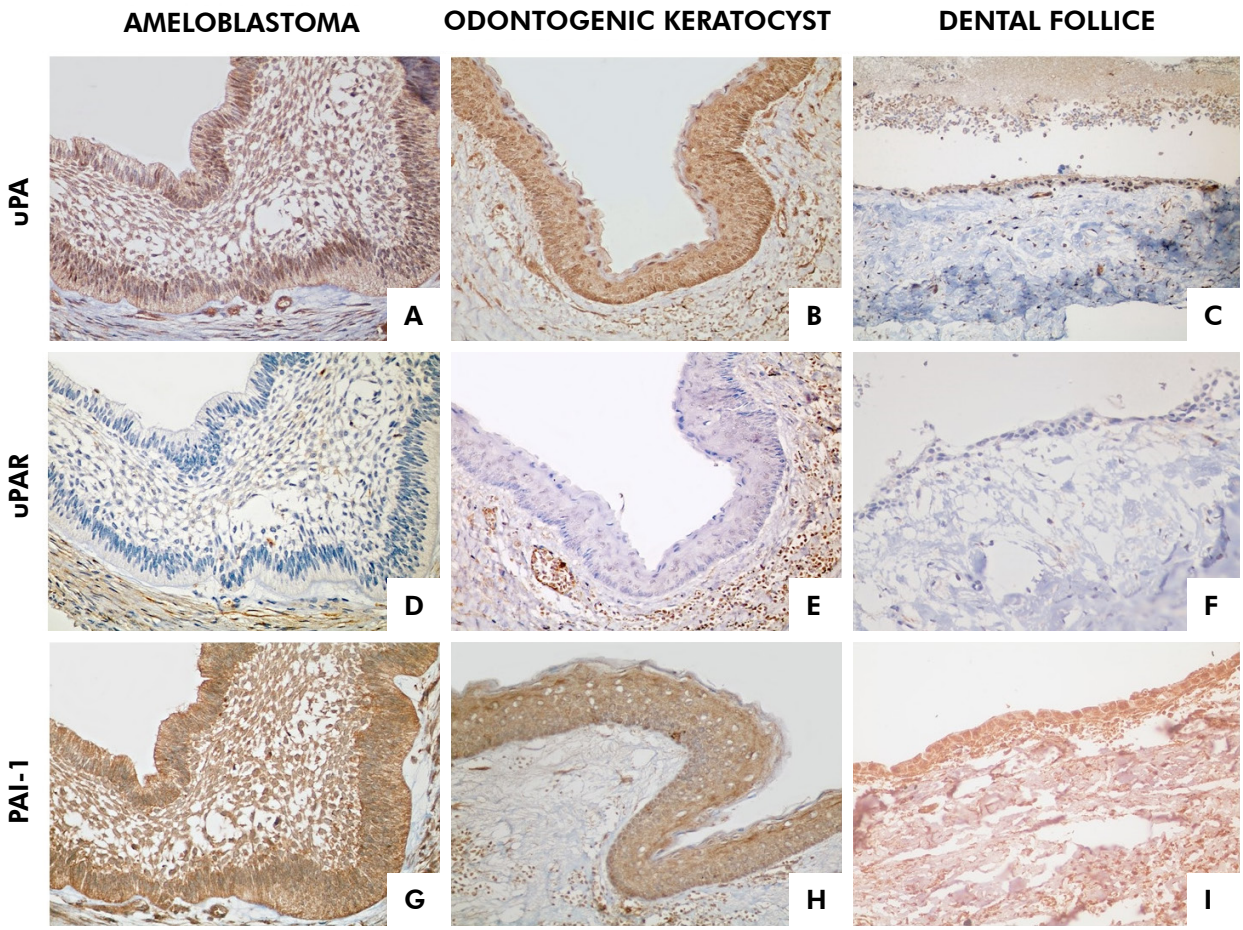

Figure 1. Immunoexpression in odontogenic epithelium. uPA: (A) indistinct staining pattern in AMB; (B) uniform staining in OKC; (C) reduced enamel epithelium of DF. uPAR: (D) low percentage of stained cells in AMB; (E) absence of immunostaining in the cystic epithelium of OKC; (F) absence of immunostaining in DF. PAI-1: (G) indistinct staining pattern in AMB; (H) uniform staining in OKC and (I) in reduced enamel epithelium of DF. Images captured at 400x magnification.

OKCs, and 60% of DFs for uPA immunostaining in connective tissue cells (Figure 2A-C). The intensity of immunopositivity was predominantly weak in AMBs and DFs and mainly moderate in OKCs. Regarding uPAR immunoexpression, all cases of OKCs and DFs, and most AMBs (85%) had up to 50% of positive connective tissue cells (Figure 2D-F). The intensity of uPAR immunopositivity was predominantly weak in all studied groups. Concerning PAI-1 immunoexpression, 45% of the AMB cases had a score of 4 (Figure 2G), 90% of the OKC cases were evenly divided between scores 3 and 4 (Figure 2H), while 50% of the DFs had a score of 2 (Figure 2I). The intensity of immunopositivity for PAI-1 was predominantly moderate in all groups. The statistical analysis showed no significant differences in uPA ($p = 0.291$), uPAR ($p = 0.056$), and PAI-1 ($p = 0.277$) immunopositivity scores (Table 5).

Table 1. Distribution of ameloblastomas, odontogenic keratocysts and dental follicles according to the epithelial scores of immunopositivity and intensity for uPA, uPAR and PAI-1.

Biomarker/ score	Group		
	Ameloblastoma	Odontogenic keratocyst	Dental follicle
	n (%)	n (%)	n (%)
uPA			
Immunopositivity			
0 (0%)	1 (5.0)	0 (0.0)	1 (10.0)
1 (1–10%)	0 (0.0)	0 (0.0)	0 (0.0)
2 (11–50%)	5 (25.0)	1 (5.0)	0 (0.0)
3 (51–80%)	6 (30.0)	1 (5.0)	0 (0.0)
4 (> 80%)	8 (40.0)	18 (90.0)	9 (90.0)
Intensity			
Absent	1 (5.0)	0 (0.0)	1 (10.0)
Weak	15 (75.0)	9 (45.0)	5 (50.0)
Moderate	4 (20.0)	11 (55.0)	4 (40.0)
Strong	0 (0.0)	0 (0.0)	0 (0.0)
uPAR			
Immunopositivity			
0 (0%)	2 (10.0)	15 (75.0)	6 (60.0)
1 (1–10%)	14 (70.0)	5 (25.0)	0 (0.0)
2 (11–50%)	2 (10.0)	0 (0.0)	1 (10.0)
3 (51–80%)	0 (0.0)	0 (0.0)	2 (20.0)
4 (> 80%)	2 (10.0)	0 (0.0)	1 (10.0)
Intensity			
Absent	2 (10.0)	15 (75.0)	6 (60.0)
Weak	14 (70.0)	3 (15.0)	4 (40.0)
Moderate	4 (20.0)	1 (5.0)	0 (0.0)
Strong	0 (0.0)	1 (5.0)	0 (0.0)
PAI-1			
Immunopositivity			
0 (0%)	0 (0.0)	0 (0.0)	0 (0.0)
1 (1–10%)	0 (0.0)	0 (0.0)	0 (0.0)
2 (11–50%)	0 (0.0)	0 (0.0)	0 (0.0)
3 (51–80%)	1 (5.0)	1 (5.0)	0 (0.0)
4 (> 80%)	19 (95.0)	19 (95.0)	10 (100.0)
Intensity			
Absent	0 (0.0)	0 (0.0)	0 (0.0)
Weak	4 (20.0)	5 (25.0)	1 (10.0)
Moderate	14 (70.0)	10 (50.0)	6 (60.0)
Strong	2 (10.0)	5 (25.0)	3 (30.0)
Total	20 (100.0)	20 (100.0)	10 (100.0)

Table 2. Parameters used for the calculation of the Kruskal-Wallis (KW) test for the evaluation of the epithelial scores of immunoexpression of uPA, uPAR, and PAI-1 in ameloblastomas, odontogenic keratocysts and dental follicles.

Biomarker/ group	n	Median	Q_{25}–Q_{75}	Mean of ranks	KW	p-value
uPA						
Ameloblastoma	20	3.00	2.00–4.00	18.25	12.657	0.002*
Odontogenic keratocyst	20	4.00	4.00–4.00	30.58		
Dental follicle	10	4.00	4.00–4.00	29.85		
uPAR						
Ameloblastoma	20	1.00	1.00–1.00	33.60	14.847	0.001*
Odontogenic keratocyst	20	0.00	0.00–0.75	17.25		
Dental follicle	10	0.00	0.00–3.00	25.80		

*Statistically significant results.

Table 3. Parameters used for the calculation of the Mann-Whitney (*U*) test for the evaluation of the epithelial scores of immunoexpression of uPA and uPAR in ameloblastomas, odontogenic keratocysts and dental follicles.

Biomarker/ group	n	Median	Q_{25}–Q_{75}	Mean of ranks	U	p-value
uPA						
Ameloblastoma	20	3.00	2.00–4.00	15.48	99.50	0.001*
Odontogenic keratocyst	20	4.00	4.00–4.00	25.53		
Ameloblastoma	20	3.00	2.00–4.00	13.28	55.50	0.029*
Dental follicle	10	4.00	4.00–4.00	19.95		
Odontogenic keratocyst	20	4.00	4.00–4.00	15.55	99.00	0.933
Dental follicle	10	4.00	4.00–4.00	15.40		
uPAR						
Ameloblastoma	20	1.00	1.00–1.00	27.50	60.00	< 0.001*
Odontogenic keratocyst	20	0.00	0.00–0.75	13.50		
Ameloblastoma	20	1.00	1.00–1.00	16.60	78.00	0.302
Dental follicle	10	0.00	0.00–3.00	13.30		
Odontogenic keratocyst	20	0.00	0.00–0.75	14.25	75.00	0.173
Dental follicle	10	0.00	0.00–3.00	18.00		

*Statistically significant results.

Correlations between uPA, uPAR, and PAI-1 expression

Spearman's correlation test (*r*) revealed the absence of significant correlations between epithelial immunoexpression scores for uPA and uPAR in AMBs (r = 0.322; p = 0.166), OKCs (r = -0.173; p = 0.466), and DFs (r = 0.262; p = 0.464). Similarly, no significant correlations were found between uPA and PAI-1 expression in AMBs (r = 0.399; p = 0.081) or OKCs (r = -0.076; p = 0.749). The correlations between uPAR and PAI-1 were also not significant in AMBs (r = 0.049; p = 0.837) or OKCs (r = -0.397; p = 0.083). Given that no variation in PAI-1 immunopositivity scores was observed in DFs, it was not possible to correlate the expression of this protein with uPA or uPAR.

As with the correlation test for epithelial immunoexpression, Spearman's correlation test (r) revealed no significant correlations between uPA and uPAR connective tissue immunoexpression scores in AMBs (r = 0.171; p = 0.470), OKCs (r = 0.100; p = 0.676), and DFs (r = 0.448; p = 0.194). The correlations between uPAR and PAI-1 were also not significant in AMBs (r = 0.251; p = 0.286), OKCs (r = -0.352; p = 0.128), or DFs (r = -0.136; p = 0.709). Similarly, no significant correlations were found between uPA and PAI-1 expression in AMBs (r = 0.425; p = 0.061), OKCs (r = 0.091; p = 0.703), and DFs (r = -0.149; p = 0.680).

The analysis of the correlation between the immunoexpression levels of each protein in the epithelium and connective tissue is described in Table 6. In AMBs, the correlation test revealed a strong positive

Table 4. Distribution of ameloblastomas, odontogenic keratocysts and dental follicles according to the connective tissue scores of immunopositivity and intensity for uPA, uPAR and PAI-1.

Biomarker/ score	Group		
	Ameloblastoma	Odontogenic keratocyst	Dental follicle
	n (%)	n (%)	n (%)
uPA			
Immunopositivity			
0 (0%)	1 (5.0)	0 (0.0)	0 (0.0)
1 (1–10%)	2 (10.0)	1 (5.0)	2 (20.0)
2 (11–50%)	10 (50.0)	10 (50.0)	6 (60.0)
3 (51–80%)	4 (20.0)	7 (35.0)	2 (20.0)
4 (> 80%)	3 (15.0)	2 (10.0)	0 (0.0)
Intensity			
Absent	1 (5.0)	0 (0.0)	0 (0.0)
Weak	13 (65.0)	7 (35.0)	7 (70.0)
Moderate	6 (30.0)	13 (65.0)	3 (30.0)
Strong	0 (0.0)	0 (0.0)	0 (0.0)
uPAR			
Immunopositivity			
0 (0%)	0 (0.0)	0 (0.0)	2 (20.0)
1 (1–10%)	8 (40.0)	13 (65.0)	5 (50.0)
2 (11–50%)	9 (45.0)	7 (35.0)	3 (30.0)
3 (51–80%)	1 (5.0)	0 (0.0)	0 (0.0)
4 (> 80%)	2 (20.0)	0 (0.0)	0 (0.0)
Intensity			
Absent	0 (0.0)	0 (0.0)	2 (20.0)
Weak	15 (75.0)	18 (90.0)	8 (80.0)
Moderate	5 (25.0)	2 (10.0)	0 (0.0)
Strong	0 (0.0)	0 (0.0)	0 (0.0)
PAI-1			
Immunopositivity			
0 (0%)	0 (0.0)	0 (0.0)	0 (0.0)
1 (1–10%)	1 (5.0)	0 (0.0)	0 (0.0)
2 (11–50%)	4 (20.0)	2 (10.0)	5 (50.0)
3 (51–80%)	6 (30.0)	9 (45.0)	2 (20.0)
4 (> 80%)	9 (45.0)	9 (45.0)	3 (30.0)
Intensity			
Absent	0 (0.0)	0 (0.0)	0 (0.0)
Weak	8 (40.0)	0 (0.0)	1 (10.0)
Moderate	11 (55.0)	13 (65.0)	7 (70.0)
Strong	1 (5.0)	7 (35.0)	2 (20.0)
Total	20 (100.0)	20 (100.0)	10 (100.0)

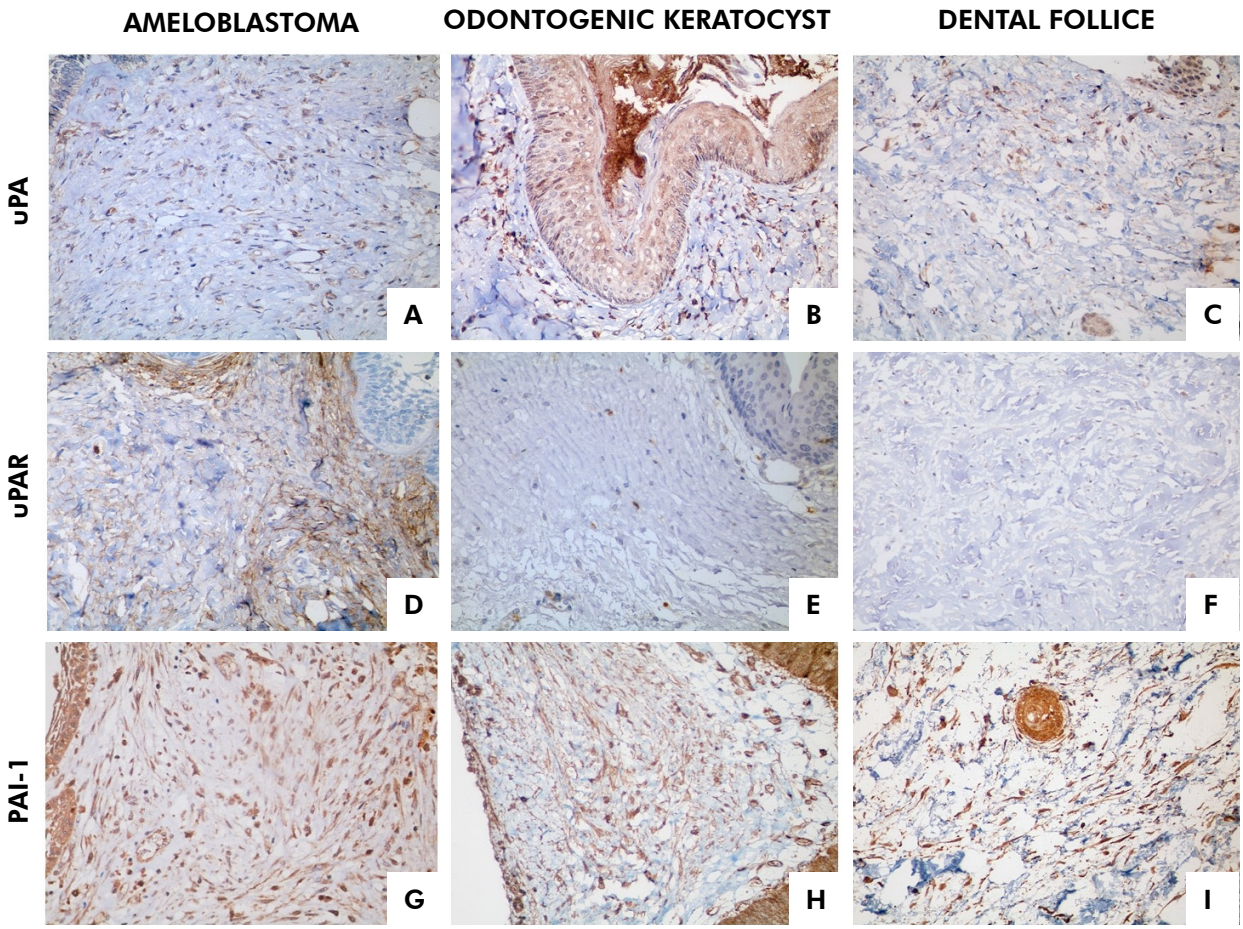

Figure 2. Immunohistochemical expression in connective tissue. uPA: (A) weak stromal staining in ameloblastoma; (B) moderate capsule staining in odontogenic keratocyst; (C) weak staining in dental follicle. uPAR: (D) moderate immunostaining in ameloblastoma; (E) low percentage of stained cells in odontogenic keratocyst; (F) few stained cells in dental follicle. PAI-1: (G) strong immunostaining in ameloblastoma; (H) moderate to strong staining in odontogenic keratocyst; (I) stained cells in dental follicle. Images captured at 400x magnification.

and statistically significant correlation between the epithelial and connective tissue immunoexpression of uPAR ($r = 0.582$; $p = 0.007$), in addition to a tendency towards a moderate positive correlation between PAI-1 immunostaining in epithelial and connective tissue cells ($r = 0.404$; $p = 0.077$), according to Cohen's classification.[18] Nevertheless, no significant correlation was found in uPA immunoexpression. Regarding OKCs and DFs, there was no correlation between epithelial and connective tissue immunoexpression for uPA, uPAR, and PAI-1. Because no variation in PAI-1 epithelial immunopositivity scores was observed in DFs, it was not possible to correlate the scores of epithelial and connective tissue immunoexpression of this protein.

Discussion

The PAS has attracted the attention of researchers because of its wide range of targets and its role in the control of various biological events, such as ECM degradation, which are critical for tumorigenesis.[10] Few studies, however, have investigated the role of the PAS in odontogenic lesions, including investigations of ameloblastic tumors, OKCs, dentigerous cysts, and radicular cysts.[11-13]

Kumamoto and Ooya[11] studied the role of ECM remodeling in odontogenic tumors. The authors observed a predominance of uPA immunopositivity in peripheral cells of the tumor islands of AMBs, but

Table 5. Parameters used for the calculation of the Kruskal-Wallis (KW) test for the evaluation of the scores of connective tissue immunoexpression of uPA, uPAR, and PAI-1 in ameloblastomas, odontogenic keratocysts, and dental follicles.

Biomarker/ group	n	Median	Q_{25}–Q_{75}	Mean of ranks	KW	p-value
uPA						
Ameloblastoma	20	2.00	2.00–3.00	25.20	2.467	0.291
Odontogenic keratocyst	20	2.00	2.00–3.00	28.40		
Dental follicle	10	2.00	1.75–2.25	20.30		
uPAR						
Ameloblastoma	20	2.00	1.00–2.00	30.65	5.771	0.056
Odontogenic keratocyst	20	1.00	1.00–2.00	23.38		
Dental follicle	10	1.00	0.75–2.00	19.45		
PAI-1						
Ameloblastoma	20	3.00	2.25–4.00	25.75	2.567	0.277
Odontogenic keratocyst	20	3.00	3.00–4.00	28.15		
Dental follicle	10	2.50	2.00–4.00	19.70		

*Statistically significant results.

Table 6. Spearman's correlation coefficients (*r*) to test for correlations between uPA, uPAR and PAI-1 immunoexpression scores in ameloblastomas, odontogenic keratocysts and dental follicles.

Correlation	Ameloblastoma		Odontogenic keratocyst		Dental follicle	
	r	p-value	r	p-value	r	p-value
uPA (Ep) x uPA (CT)	0.357	0.123	0.253	0.282	0.527	0.117
uPAR (Ep) x uPAR (CT)	0.582	0.007*	-0.182	0.444	0.357	0.311
PAI-1 (Ep) x PAI-1 (CT)	0.404	0.077	-0.242	0.304	-	-

* Statistically significant results. - It was not possible to perform a statistical test; Ep: epithelial immunopositivity score; CT: connective tissue immunopositivity score.

not in areas of squamous metaplasia or granular differentiation. By contrast, our study revealed high immunoexpression of uPA both in peripheral and in central cells. Additionally, areas of squamous metaplasia exhibited higher staining intensity for this protein, while the immunoexpression pattern in granular cells was similar to that of adjacent cells. Similarly to the AMB cases, OKCs exhibited uniform staining for uPA throughout the cystic epithelium, except for cases in which the parakeratin layer was more intensely stained, suggesting involvement of this protein in cell differentiation.

Epithelial immunopositivity for uPA was observed in normal odontogenic tissues represented by DFs, in agreement with the findings of Kumamoto and Ooya,[11] who studied tooth germs. In addition, epithelial immunoexpression of uPA was lower in AMBs than in OKCs and DFs, suggesting the interaction between uPA and uPAR is not a determinant factor for the infiltrative growth of AMB.

Kubota et al.[13] studied the regulation of MMP-9 secretion and activation by interleukin-1α (IL-1α) in odontogenic cysts. These authors found that IL-1α may upregulate not only proMMP-9 secretion, but also proMMP-9 activation by inducing proMMP-3 and uPA production in OKC epithelial cells. According to the authors, these findings could be explained because plasminogen is converted by uPA to plasmin which, in turn, activates proMMP-3. Subsequently, the activated MMP-3 activates proMMP-9. These results suggest the studied proteins, including uPA, may play a role in OKC expansion.

The epithelial immunoexpression of uPA was similar in OKCs and DFs, probably because the DF covers the crown of impacted teeth and this protein is thus expressed to assist in ECM degradation during tooth eruption. The significantly higher epithelial immunoexpression in OKCs as compared to AMBs suggests uPA is more related to cystic growth than to tumor growth. In the study of Serratì et al.,[12] uPA

was detected by enzyme immunoassay (ELISA) in the cystic fluid of dentigerous and radicular cysts, with higher levels in radicular cysts, probably because of the associated inflammatory component.

Regarding uPAR, we observed low epithelial immunopositivity in AMBs, while most OKC and DF cases were negative for this protein. In the study of Kumamoto and Ooya,[11] uPAR was immunoexpressed mainly in peripheral cells of the follicular and plexiform structures of AMBs, but not in acanthomatous areas or granular cells. On the other hand, when studying oral squamous cell carcinoma (OSCC), Magnussen et al.[17] observed higher immunoexpression of uPAR in tumor cells with a high degree of differentiation. This observation is consistent with the findings of the present study, in which higher uPAR staining was found in areas of squamous and granular differentiation. We therefore suggest uPA and uPAR are associated with epithelial cell differentiation.

In the sample studied, epithelial immunoexpression of uPAR did not differ significantly between AMBs and DFs. Kumamoto and Ooya[11] also found similar immunoexpression of uPAR when AMBs and tooth germs were compared. However, these authors observed immunopositivity for this protein in all tooth germs analyzed, while this protein was not expressed in 60% of DFs analyzed in the present study. In addition, our study showed higher epithelial immunoexpression of uPAR in AMBs when compared to OKCs. This finding suggests that, in addition to binding to uPA to convert plasminogen to plasmin, this receptor may interact with other receptors, integrins and ECM components in AMBs, transducing intracellular signals related to cell proliferation, survival, and migration, thus contributing to tumorigenesis.[5,6,19]

In contrast to our results, Serratì et al.[12] showed strong immunoexpression of uPAR in the cystic epithelium of dentigerous and radicular cysts. This finding might be explained by the fact that the antibody employed by these authors recognizes both the intact and cleaved form of uPAR (DII-DIII), while the antibody used here can only bind to the intact form of the receptor. The cleaved forms of uPAR predominated in the cystic fluid, probably because of the high concentration of uPA, which catalyzes the proteolytic cleavage of the receptor.[12] We therefore highlight the presence of uPAR in cleaved and soluble form, which can act as chemoattractants and induce cell migration.[20,21] Within this context, in an *in vitro* study on OSCC, Magnussen et al.[22] showed that part of uPAR is present in cleaved form [uPAR (DII-DIII)], mainly as a result of the action of uPA and plasmin. The authors also observed that overexpression of PAI-1 resulted in reduced cleavage of uPAR and lower cell migration. In addition, enzymatic inhibition of uPA reduced cell invasion. According to these authors, the cleaved forms of uPAR are also involved in invasion and metastasis.[22] In the present study, it was not possible to evaluate the cleaved and soluble forms of uPAR because the anti-uPAR antibody used for the immunohistochemical analysis only detects the intact form of the receptor. Thus, further studies are needed to elucidate the role of the cleaved and soluble forms of uPAR in the pathogenesis of AMBs and OKCs.

In a study on OSCC, Christensen et al.[23] observed highly specific expression of uPAR in tumor tissue, while expression was scarce in the surrounding oral mucosa. Serpa et al.[24] found an association between uPAR immunoexpression in SCC of the tongue and locoregional recurrence, suggesting this protein plays a role in tumor aggressiveness. Thus, studies analyzing the relationship of uPAR immunoexpression with recurrence in AMBs and OKCs could support the choice of treatment for these lesions. Accordingly, in the study of Gao et al.,[25] silencing of uPAR in SCC of the tongue downregulated MMP expression, reduced *in vitro* cell invasion, and activated the ERK/MAPK signaling pathway, which is important for these processes. The authors also observed that silencing of uPAR reduced the incidence of pulmonary metastases *in vivo*. Within this context, we suggest the higher epithelial expression of uPAR in AMB contributes to the local invasion potential of this tumor.

The present study demonstrated the absence of significant differences in the immunoexpression of PAI-1 between AMBs and OKCs. Nonetheless, the high percentage of positive epithelial cells suggests a possible role for this protein in the development of AMBs and OKCs. When studying odontogenic

cysts, Serratì et al.[12] observed lower concentrations of PAI-1 in the cystic fluid of radicular cysts when compared to dentigerous cysts. According to these authors, this finding may reflect greater activation of uPA in radicular cysts proportional to the degree of inflammation.

The strong epithelial immunoexpression of PAI-1 in AMBs and DFs observed in the present study is consistent with the findings of Kumamoto and Ooya,[11] who analyzed AMBs and tooth germs. In AMBs, these authors found lower PAI-1 immunoexpression in areas of squamous metaplasia as compared to adjacent cells, in addition to the absence of staining in granular cells. However, in the present study, the immunoexpression of this protein in areas of squamous/granular differentiation was similar to that found in the other neoplastic cells. Similarly, most OKCs exhibited diffuse epithelial expression of PAI-1. By contrast, Peterle et al.[26] found an association between PAI-1 overexpression and a low grade of cell differentiation in OSCCs. Taken together, these findings suggest that the functions of PAI-1 depend on the type of lesion and that this protein may not be related to epithelial cell differentiation in AMBs or OKCs.

Differently from what is seen in odontogenic lesions, in OSCC, the expression of PAI-1 is significantly higher in tumor cells than in normal tissues.[27,28] In OSCC, high expression of uPA and PAI-1 were associated with a higher recurrence rate. Patients with low levels of PAI-1 treated only with surgical excision had a lower probability of recurrence when compared to cases with high PAI-1 levels.[27] Furthermore, low immunoexpression of PAI-1 was reported to be associated with greater disease-free survival.[17]

Despite the protumor and antitumor functions exerted by PAI-1, the analyzed sample showed no significant differences between DFs (physiological tissue) and the studied lesions (AMB and OKC). The antitumor functions of PAI-1 are related to its uPA inhibitory function and, consequently, to plasmin formation, thus reducing ECM remodeling, an important process for tumor progression and invasion. In turn, protumor function is related to increased proliferative activity and resistance to apoptosis, in addition to contributing to angiogenesis, migration, and cell invasion through activation of signaling pathways such as MAPK and JAK/STAT.[4,6,7,26] However, the high percentage of PAI-1-positive epithelial cells in AMBs, OKCs, and DFs suggests a possible role in its development. We can assume that in AMBs and OKCs, PAI-1 is related to its protumor functions, while in DFs, it would probably be related to antitumor functions, in a phase before tooth eruption, contributing to the non-remodeling of ECM and protecting the tooth germ.

Although we did not find statistically significant differences between the immunoexpression levels of uPA, uPAR, and PAI-1 in the studied groups, the expression of these proteins in the stroma of AMBs and in the capsule of OKCs suggests their importance in the infiltrative growth of these lesions. Kumamoto and Ooya[11] found predominance of uPA expression in the stroma and uPAR expression in epithelial neoplastic cells in ameloblastic tumors. Differently from these results, our study found greater staining of uPA in epithelial cells and uPAR in connective tissue cells, both in AMBs and in OKCs. Regarding PAI-1, as evidenced in our study, Kumamoto and Ooya[11] found similar PAI-1 labeling in both cell types in the studied lesions. The stromal immunoexpression of uPA, uPAR, and PAI-1 has also been described in OSCC.[17,20,22]

As uPA, uPAR, and PAI-1 are part of the same enzyme system, they are connected and the balance in the expression of these proteins will indicate the effects they will exert. Within this context, studies on OSCC reported significant correlations between the expression levels of these molecules in tumor and normal tissues, suggesting the concentrations of these factors help regulate tumor invasion and metastasis.[27,28] Nevertheless, we found no correlation between epithelial or connective tissue immunoexpression of uPA, uPAR, and PAI-1 in the studied groups, a finding that might be explained by the small size of the sample. However, we evidenced a strong correlation between the epithelial and connective tissue immunoexpression of uPAR in AMBs. This finding suggests that increases in the expression of uPAR and PAI-1 in epithelial and connective tissue cells in AMBs are related to tumorigenesis. Through the PAS and MMP activation, the binding of

uPAR to uPA acts on the degradation of the ECM. In addition, through the interaction with other molecules, integrins, receptors, and components of the ECM, uPAR, and PAI-1 can act on cell proliferation, survival, and angiogenesis, processes that are necessary for tumorigenesis. To our knowledge, there are no studies in the literature that have established this correlation in odontogenic cysts and tumors.

Conclusion

In summary, the high epithelial expression of uPA in OKCs suggests the participation of this protein in the pathogenesis of these lesions by contributing to ECM degradation. The higher epithelial immunoexpression of uPAR in AMBs indicates involvement of this protein in tumorigenesis, possibly activating signaling pathways that participate in processes such as cell proliferation, migration, and survival. The high percentage of PAI-1-positive cells suggests a possible role for this protein in the development of AMBs and OKCs.

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