



Comparison of immunoexpression of VEGF, TGF- β and MMP-9 in ameloblastoma and adenomatoid odontogenic tumor

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Abstract

Purpose: Studies on odontogenic tumors have identified various molecular dysfunctions involved on their development, and some mechanisms such as angiogenesis and matrix modulation, are useful means of investigating the differences in biologic behavior of these tumors. Some important markers to identify tumor aggressiveness by immunohistochemistry are VEGF, TGF- β and MMP-9 proteins. This study aimed to compare the immunohistochemical expression of VEGF, TGF- β , and MMP-9 in ameloblastoma and adenomatoid odontogenic tumor (AOT).

Methods: Immunoexpression of VEGF, TGF- β , and MMP-9 was studied in 15 solid ameloblastomas, and 15 AOTs. A semi-quantitative analysis of the immunostained cells was performed, and the statistical analysis was made using the Mann-Whitney nonparametric and Spearman correlation test, with significance level at .05 ($P < .05$).

Results: A higher epithelial immunoexpression of VEGF and TGF- β was observed in ameloblastoma and AOT, respectively, and the stromal reactivity to VEGF was statistically higher in ameloblastoma ($P < .05$). No statistical difference was observed for MMP-9 ($P > .05$).

Conclusion: The results suggest the involvement of angiogenesis in tumor progression of ameloblastomas, and the inductive effect of stromal cells in AOT, hence justifying its lower growth potential.

Key words: Odontogenic tumors; Immunohistochemistry; Vascular endothelial growth factor (VEGF); Matrix metalloproteinase 9 (MMP-9); Transforming growth factor β (TGF- β)

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Comparação da imunoexpressão de VEGF, TGF- β e MMP-9 em ameloblastoma e tumor odontogênico adenomatóide

Resumo

Objetivo: Estudos sobre tumores odontogênicos têm identificado várias disfunções moleculares envolvidas no seu desenvolvimento, e alguns mecanismos como a angiogênese e modulação da matriz são objetos úteis para investigar as diferenças no comportamento biológico destes tumores. Alguns marcadores importantes para identificar a agressividade do tumor por imunoistoquímica são as proteínas VEGF, TGF- β e MMP-9. Este estudo teve como objetivo comparar a expressão imunoistoquímica de VEGF, TGF- β e MMP-9 entre ameloblastoma e tumor odontogênico adenomatóide (TOA).

Métodos: Imunoexpressão de VEGF, TGF- β e MMP-9 foi estudada em 15 ameloblastomas sólidos e 15 TOA. Uma análise semiquantitativa das células imunomarcadas foi realizada e a análise estatística foi feita usando o teste não paramétrico de Mann-Whitney e o teste de correlação de Spearman, com nível de significância de 0,05 ($P < 0,05$).

Resultados: Maior imunoexpressão epitelial de VEGF e TGF- β foi observada em ameloblastoma e TOA, respectivamente; a reatividade do estroma para VEGF foi estatisticamente maior no ameloblastoma ($P < .05$). Não houve diferença estatística para MMP-9 ($P > 0,05$).

Conclusão: Os resultados sugerem o envolvimento da angiogênese na progressão tumoral de ameloblastomas e o efeito indutor de células estromais em TOA, portanto, justificando o seu potencial de crescimento mais baixo.

Palavras-chave: Tumores odontogênicos; Imunoistoquímica; Fator de crescimento endotelial vascular (VEGF); Metaloproteinase da matriz 9 (MMP-9); Fator de transformação do crescimento β (TGF- β)

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Introduction

Ameloblastoma is an aggressive benign epithelial odontogenic tumor that represents about 1% of all tumors and cysts of the jaws. It is considered the most clinically significant odontogenic tumor [1]. Although regarded as a benign neoplasm, it is locally invasive with frequent recurrence and large destruction of the jaws even following radical surgery [2].

Adenomatoid Odontogenic Tumor (AOT) is an uncommon tumor of odontogenic origin that represents only 3% of all odontogenic tumors. AOT is histologically characterized by the presence of duct-like structures with amyloid-like deposits [3]. Although comprised of odontogenic epithelium embedded in a matured connective tissue stroma, AOT commonly has an indolent evolution what allows it to be efficiently treated by either conservative surgical enucleation or curettage [4].

Vascular endothelial growth factor (VEGF) is a sub-family of growth factors which encode important signaling proteins involved in both vasculogenesis (the *de novo* formation of the embryonic circulatory system), and angiogenesis (the growth of blood vessels from pre-existing vasculature) [5]. This growth factor also plays an important role in restoring the oxygen supply to tissues when blood circulation is inadequate [6]. Transforming growth factor-beta (TGF- β) regulates pivotal cellular processes such as proliferation, differentiation, and apoptosis [7]. The cellular distribution of TGF- β receptors along with their oligomerization mode and complex interaction with different cell surface receptors is a crucial requirement for the initiation of distinct signaling cascades [8]. This protein also inhibits degradation of the extracellular matrix both through an inhibitory action of the production of matrix metalloproteinase, and a stimulatory action that activates enzyme inhibitors [8]. Matrix metalloproteinase-9 (MMP-9) is a type IV collagenase that plays an active role not only in promoting tumor cell invasion and metastasis, but also as a marker of the tumor invasive phenotype. Elevated MMP-9 expression in malignant epithelial and stromal cells has been found in tumors of the breast, lung, prostate, and pancreas [3]. Expression of VEGF, MMP-9, and TGF- β has been investigated in odontogenic tumors, thereby suggesting that they may play a role in the regulation of tumor growth [9].

The aim of the present study was to compare the immunohistochemical expression of VEGF, TGF- β , and MMP-9 in ameloblastoma and AOT to provide a better understanding of the biological behavior of these two distinct tumors.

Methods

This research was reviewed and approved by the Research Ethics Committee of the Federal University of Rio Grande do Norte.

The sample was obtained from the pathologic anatomy lab of the oral pathology department at Federal University of Rio Grande do Norte. It consisted of 15 cases of solid ameloblastomas, and 15 AOTs, all diagnosed according to the World Health Organization (WHO) classification.

The selected material was fixed in formalin at 10%, and later embedded in paraffin. Histologic sections of 3- μ m thick were extended on glass slides containing a 3-aminosilane-propyltriethoxy-based adhesive (Sigma Chemical CO, St. Louis, MO, USA). They were dewaxed in two xylene baths, the first being at 60°C for 30 min, and the second at room temperature for 20 min, and then re-hydrated before being washed in two passages through distilled water for 5 min each. Endogenous peroxidase was inactivated by using hydrogen peroxide 10 vol. The sections were subsequently washed in water, and immersed twice for 5 min in a buffer solution of Tris (hydroxymethyl) aminomethane (TRIS-HCL), pH 7.4. After this, the slides were incubated for 30 min at room temperature with antibodies diluted in TRIS-HCL buffer solution (Table 1) using the streptavidin-biotin complex (Dako-Cytomation LSAB+System-HRP, Dakocytomation, A/S, Glostrup, Denmark). The revelation was made in a dark room by adding for 3 min the chromogenic agent diaminobenzidine which had been diluted in a solution containing TRIS-HCL and hydrogen peroxide 10 vol. The samples were counterstained with Mayer hematoxylin for 10 min, and then rinsed with water after the end of each step. Finally, dehydration in ethanol and xylene clearing were both carried out to mount the glass slides with Erv-mount.

For the immunohistochemical analysis, three examiners were in charge of verifying at different times either the presence or absence of VEGF, TGF- β and MMP-9 expression in both epithelial and stromal cells. A semi-quantitative analysis of the immunostained cells was performed using scores adapted from Florez-Moreno et al. [10]. Regardless of immunohistochemical staining, the tumor largest area in each side was elected at 100x magnification. Eight randomly selected histological fields were then photographed at a 400x magnification using an OLYMPUS® light microscope with a coupled digital camera. The images were transferred to a computer with the OLYMPUS Master v.1.41 EX system, and the cells were counted with the aid of the IMAGE J® software. First, in each field, positive and negative cells were counted, and the following scores in each sample

Table 1. Monoclonal antibodies and staining conditions.

Clone	Specification	Source	Dilution	Incubation time	Antigen retrieval
SP28	VEGF	LSAB	1:500	Overnight (18 h)	Trypsin 0.1%
sc-146	TGF- β	LSAB	1:700	60 min	Citrate pH 6.0 Pa
2C3	MMP-9	Novocastra	1:20	Overnight (18 h)	Citrate pH 6.0 Pa

were applied: 0 (no one positive tumor cells), 1 (10-25% of positive tumor cells), 2 (25-50% of positive tumor cells), 3 (50-75% of positive tumor cells) and 4 (>75% of positive tumor cells). After obtaining the data, a descriptive analysis of the results was carried out.

Comparative analysis of data was performed using the Mann-Whitney nonparametric test. Spearman correlation test was performed to verify possible correlations of immunoexpression between the tumors. For all tests, the significance level was set at .05 ($P < .05$).

Results

The epithelial immunoreactivity to VEGF, TGF-β and MMP-9 is shown in Table 2. No statistical difference was observed for MMP-9 ($P > .05$). However, a higher immunoexpression of VEGF and TGF-beta was observed in ameloblastoma and AOT, respectively ($P < .05$).

In regard to stromal cells, it was possible to observe the presence of positive fibroblasts-like, endothelial-like, polymorphonuclear neutrophil-like, plasma cell-like, lymphocyte-like, and macrophage-like cells. Table 3 summarizes the analysis of antibody staining in the stromal cells. The median reactivity to VEGF was statistically higher in ameloblastoma than in AOT ($P < .05$).

Table 2. Mann-Whitney U test for the score assessment of epithelial cells immunostained by MMP-9, VEGF and TGF-β, according to the type of lesion.

Marker	Type of lesion	N	Median	Mean of ranks	U	P value
MMP-9	AML	15	3	16.37	99.5	0.517
	AOT	15	3	14.63		
VEGF	AML	15	3	22.43	8.5	0.000
	AOT	15	1	8.57		
TGF-β	AML	15	1	8.40	6.0	0.000
	AOT	15	3	22.60		

AML – ameloblastoma; AOT – adenomatoid odontogenic tumor.

Table 3. Mann-Whitney U test for the score assessment of stromal cells immunostained by MMP-9, VEGF and TGF-β, according to the type of lesion.

Marker	Type of lesion	N	Median	Mean of ranks	U	P value
MMP-9	AML	15	3	16.37	99.5	0.517
	AOT	15	3	14.63		
VEGF	AML	15	4	23.0	0	0.000
	AOT	15	1	8.0		
TGF-β	AML	15	1	15.50	112.5	1.000
	AOT	15	1	15.50		

AML – ameloblastoma; AOT – adenomatoid odontogenic tumor.

The Spearman correlation test showed no significant correlation between the studied proteins in ameloblastoma and AOT.

Discussion

Odontogenic tumors comprise a group of lesions of the jaws, derived from primordial tooth-forming tissues, presenting in a large number of histologic patterns and behaving quite differently [11]. Although ameloblastoma is a benign neoplasm, it normally has a locally aggressive evolution. AOT, in the other hand, has an indolent growth, thereby requiring a more conservative approach [9].

VEGF plays a pivotal role in neovascular formation. Its inhibition by using siRNA significantly decreased tumor vascularity as well as reduced tumor-induced bone lysis [1]. Studies have demonstrated the importance of VEGF in chemotaxis, osteoclast migration, invasion, and activation, hence supporting the survival of mature osteoclasts [5,6]. VEGF also up-regulates the expression of RANK and increases the angiogenic response of endothelial cells to RANKL [12]. These processes play a key role in repairing and remodeling bone during development and, in odontogenic tumors, they promote bone resorption [13]. Numerous observations have shown that VEGF is generated from a variety of cells inside or in the close vicinity of blood vessels under many physiological or pathological conditions and it acts as a major player in the initial step of angiogenesis [13].

In the present study, the immunoexpression of VEGF was more evident in epithelial and stromal cells of ameloblastoma when compared with AOT. This finding may be closely related to the angiogenesis and invasive biological behavior of ameloblastomas, corroborating the studies of Chen et al. [1] and Kumamoto et al. [13] who have already suggested that VEGF is an important mediator of angiogenesis in these epithelial odontogenic tumors. Its up-regulation might be associated with malignant neoplastic changes of odontogenic epithelial cells.

TGF-β is synthesized by many different cells including those of the odontogenic epithelium. It is secreted as a latent complex that requires enzymatic cleavage of carbohydrate groups or acidification to release the active cytokine. It is known to stimulate the production of extracellular matrix such as collagen, fibronectin, proteoglycans, and others [2]. The signaling pathway of TGF-β has been found in many cellular processes (e.g. growth, differentiation, apoptosis, homeostasis, and other functions) of both the adult organism and the developing embryo [14].

Immunohistochemical detection of TGF-β and its receptors in tooth germs and epithelial odontogenic tumors supports the hypothesis that such protein can act on epithelial cells via paracrine and autocrine mechanisms. Its altered expression, especially in subtypes of ameloblastoma and AOT, suggests a possible role in affecting the differentiation of neoplastic odontogenic epithelial cells. Its dysregulation can then result in uncontrolled growth and tumor formation [14].

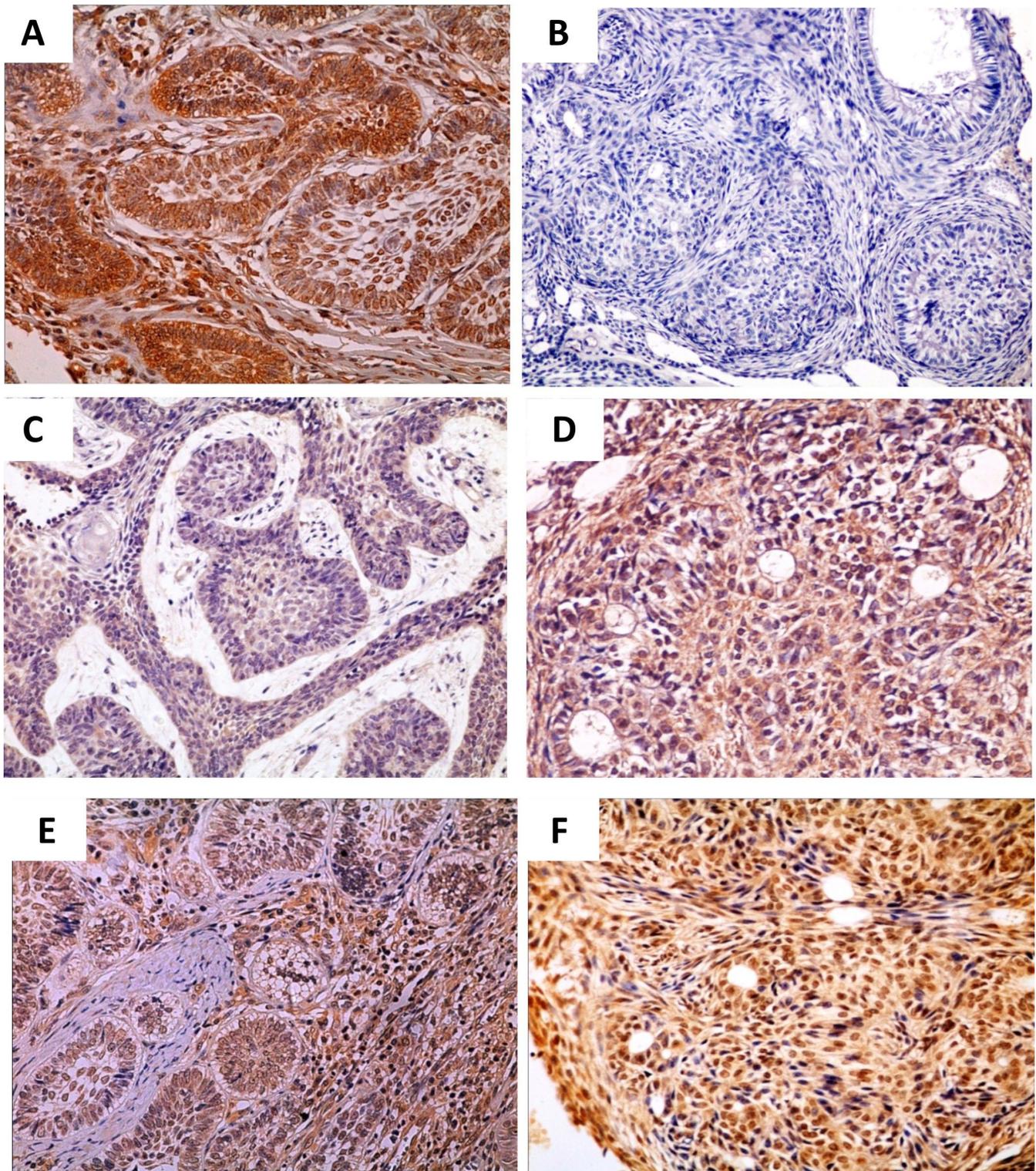


Fig. 1. Immunohistological staining of (A) VEGF in ameloblastoma, (B) VEGF in AOT, (C) TGF- β in ameloblastoma, (D) TGF- β in AOT, (E) MMP-9 in ameloblastoma, (F) MMP-9 in AOT. Original magnification $\times 100$.

In the present study, the greater cellular reactivity of AOTs to TGF- β may be due to the fact that TGF- β can stimulate the adjacent tumor stroma. This leads to an increase in the production of collagen matrix and gives origin to the characteristic capsule of AOTs which is generally

known to reduce tumor aggressiveness in comparison with ameloblastomas.

Iezzi et al. [2] compared the expression of TGF- β in ameloblastomas with different risk of recurrence. The tumors were divided into 2 groups: group A (unicystic and peripheral

ameloblastomas with a low risk of recurrence), and group B (solid ameloblastomas with a high risk of recurrence). Statistically higher values of positivity to TGF- β were observed in group B stromal cells. No statistically significant difference in immunoreactivity was found in vessels and odontogenic epithelium. The increased expression in tumors with a high risk of recurrence could be explained by the fact that, although TGF- β acts as a potent tumor suppressor in the early stages of tumor progression, later it seems to enhance the invasive phenotype of the tumor.

Takata et al. [8] investigated the immunolocalization of TGF- β in desmoplastic ameloblastoma (DA) with the aim to study its role in stromal desmoplasia. In the “hybrid” lesion, TGF- β was not found in follicular ameloblastoma, but it was in DA. These results confirm that the TGF- β produced by DA tumor cells plays a role in desmoplastic matrix formation. Kumamoto et al (5) studied the expression of TGF- β and its receptors in epithelial odontogenic tumors. They found out that the DA exhibited elevated reactivity to TGF- β and its receptors. AOT neoplastic cells also reacted to TGF- β and its receptors. Such reaction was very strong in pseudoglandular cells. Metastatic ameloblastomas showed an expression pattern similar to that seen in benign ameloblastomas.

Degradation of the extracellular components is a prerequisite for cell migration into the matrix. It represents a key element in the multistage processes of tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes that degrade extracellular matrix (ECM) macromolecules such as collagens, gelatins, fibronectin, tenascin, and laminin, at physiological pH [15]. These enzymes play a central role in the regulation of the extracellular matrix during embryonic development and tissue remodeling [3]. MMPs also participate in the extracellular matrix (ECM) destruction associated with tumor invasion and metastasis. Aberrant MMP activity in tumor cells and surrounding stromal tissues has been implicated in tumor invasion and metastasis. According to studies on *in vitro* and *in vivo* tumor invasiveness, therapeutic interventions that inhibit MMP activity appear to be very promising [16]. In the present study, no difference was observed in the expression of MMP-9 between ameloblastoma and AOT. This result does not reflect the distinct patterns of aggressiveness seen in such tumors, such as suggested by the findings of Farias et al. [17] and Henriques et al. [18].

Conclusion

In conclusion, our results suggest that the strong immunoreactivity to VEGF in ameloblastomas corroborates the involvement of angiogenesis in tumor progression, thereby contributing to increase the aggressiveness of this tumor. In the AOT, the immunoreactivity to TGF- β reflects

the inductive effect of stromal cells, hence justifying its lower growth potential.

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