Expression of CD34, VEGFR3 and eosinophil density in selected odontogenic tumors- a pilot study

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ABSTRACT

Background: Limited statistically and clinically significant studies have been down on connective tissue factors in the odontogenic tumors. Therefore, the aim of this study was to determine the biological behavior of two selected epithelial odontogenic tumors (Ameloblastoma and Adenomatoid odontogenic tumor) by detecting CD34, VEGFR3 and eosinophil densities.

Methods: In this cross-sectional study, paraffin blocks including 20 cases of ameloblastoma and 20 (AOT), were selected. Totally, 4 sections were prepared for hematoxylin-eosin, Congo red staining, immunohistochemistry with CD34 and VEGFR3. Expression of VEGFR3, CD34 and lymphatic, blood vessels and eosinophil densities was examined.

Results: The mean of blood, lymphatic vessels and eosinophils densities in ameloblastoma were 14.9 ± 6.4 , 4.4 ± 2 and 3.2 ± 2.7 , respectively; and in AOT, they were 8.9 ± 3.4 , 3.6 ± 1.3 and 1.2 ± 0.7 , respectively. There was a significant difference in eosinophils and blood vessels densities between the two lesions ($p = 0.005$; $p = 0.003$). By increasing the density of eosinophils, the density of the blood vessels increased in both lesions ($r = 0.539$, $P = 0.001$) There was no positive relationship between eosinophils and lymphatic vessels densities in the two above mentioned odontogenic lesions ($p = 0.288$, $R = 0.191$)

Conclusion: It can be suggested that tumor angiogenesis and eosinophil densities may play a more effective role than lymphangiogenesis in local invasive behavior of ameloblastoma rather than AOT.

1. Introduction

Ameloblastoma is the second most common odontogenic tumor with slow growth, including 18% of odontogenic tumors. This tumor has invasive behavior and tends to have high recurrence which rarely gives metastasis.¹

Adenomatoid odontogenic tumor is an unusual benign epithelial odontogenic tumor including 3–7% of odontogenic tumors. Due to the small size and reluctance of the tumors to recurrence, some researchers have introduced them as hamartoma. It was called pseudoadenameloblastoma with slow but progressive growth.² The treatment of these two odontogenic lesions are different; and in more aggressive treatment of ameloblastoma, marginal resection is required.² The pathologic mechanism of odontogenic tumors is closely related to the process of dental evolution, and any molecular and genetic substitution in proliferation, differentiation and apoptosis is effective in development of

the tumor and its clinical behavior.^{1,2}

Previous researchers reported high expression of MMP 2, 9, BCL2, Ki67 and decreased expression of MDM2 and P53 in invasive clinical behavior of ameloblastoma in comparison with AOT.^{1,3,4} The role of connective tissue and factors including inflammatory cells, especially eosinophils, blood and lymphatic vessels are not fully known in pathogenesis and the clinical behavior of odontogenic tumors.

Any change in the odontogenic epithelium results in changes in tumor stroma.⁴ Eosinophils are multifunctional leukocytes that are involved in the onset and progression of inflammatory, allergic, intrinsic and acquired immunity as well as tissue regeneration responses. With regards to the role and function of eosinophils, there were conflicting results in oral cancers, especially squamous cell carcinoma. It is believed that eosinophils play a role in the secretion of matrix metalloproteinases and their inhibitors in angiogenesis,⁵ but their role in the clinical behavior of benign odontogenic tumors remains unknown.

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Solid tumors are responsible for induction of angiogenesis and lymphangiogenesis through the formation of new blood vessels and lymphatic vessels. These two vessel systems have a distinct function and different structural characteristics. Their role in cancers metastasis has been investigated,⁶ but so far, limited studies has been conducted on the clinical behavior of odontogenic tumors.

Angiogenesis is the formation of new blood vessels from primary ones, which is quantitatively measured by Microvessel density as well as VEGF, CD105 and CD34 markers. CD34 is a specific marker for blood vessels, for identifying old and new formed blood vessels.⁷

VEGF is a vascular endothelial growth factor and a primary marker for angiogenesis, with 3 receptors including VEGFR1, 2 and 3, and VEGFR3 plays a major role in lymphangiogenesis, making new lymphatic vessels, and usually VEGFC and D are connected to this receptor.^{7,8}

Lymphatic vessel density (LVD) represents the relationship between tumor cells and lymphatic vessels and by them, the likelihood of invasion increases.⁸ In general, information on angiogenesis and lymphangiogenesis in tumors can help clinicians in non-invasive therapeutic methods, preventing tumor formation and recurrence, and determining tumor prognosis.⁹

Among study carried out, none has investigated eosinophils and lymphangiogenesis with VEGFR3 density in odontogenic lesions. Moreover, the relationship between blood vessels and eosinophils density in ameloblastoma and adenomatoid odontogenic tumor has not been compared. Therefore, the aim of this study was to determine the biological behavior of two selected epithelial odontogenic tumors (Ameloblastoma and Adenomatoid odontogenic tumor) by detecting CD34, VEGFR3 and eosinophil densities.

2. Materials and methods

This study was conducted with the approval of the Ethics Committee of the Research of University. In this cross-sectional study, 20 paraffin blocks of ameloblastoma (10 cases of follicular, 5 plexiform cases, 3 cases of unicystic mural and one uicystic ameloblastoma) and 20 samples of follicular and intra-osseous adenomatoid odontogenic tumors were obtained from existing paraffin blocks archives of files in dentistry faculty, and hospital– health centers.

Four sections with 5 μ dimension were prepared from the paraffin blocks for hematoxylin-eosin and Congo red staining, and immunohistochemistry with the markers, CD34 and VEGFR3.

Identification of the samples was done using H&E staining without having any information from prior identification. After removal of samples, 20 cases were confirmed with inappropriate quality and fixation as well as inadequate tissue. Clinical data of the samples (age, sex and location) were extracted from the files.

For Congo red staining, the tissue sections were deparaffinized and put in different degrees of alcohol for dehydration; and then, entered into 1% Congo red solution for 8 min. Subsequently, the tissue sections were washed and placed into KOH solution (2.5%), and then washed with the stained hematoxylin for 8 min. The tissue sections were placed in 10% alcohol. Finally, they were washed in dehydrate alcohol and dexteros, and then rubbed. To study the density of eosinophils, the stained slides were examined by an oral pathologist using optical microscope (BX41, Olympus, Tokyo, Japan) with magnification of 400 $\times$.

The only nucleate cells with cytoplasmic red granules were accepted as eosinophils. Eosinophil density was recorded in 10 microscopic fields consecutively with 400x, and then the mean was considered. Eosinophils in the regions of tumoral necrosis and degenerated muscle tissue were excluded from the study.¹⁰

2.1. Immunohistochemistry staining for blood vessels (CD34) and lymphatic vessels (VEGFR3)

At first, the paraffin blocks sections with 4 μ dimension were

deparaffined and put into various degrees of alcohol for dehydration; and then for endogenous peroxidase activity, the block was placed in 3% hydrogen peroxide and subsequently in the phosphate buffer.

The antigen was recycled in the microwave oven (Panasonic, 2001) with 2 atmospheric pressure at 120 °C for 10 min; then, it was incubated in the moist heat and with Anti VEGFR3 (mouse monoclonal Antibody VEGFR3 Product Code: NCL-L-VEGFR3, Clone: KLT, Ig, class. IgG 2b. kappa, N.Va costar TM Liquid) and Anti CD34 (mouse monoclonal antibody CD34, 9 clone Q Bend 10; Dakopatts, Glostrup, Denmark) with dilution of one fiftieth for 30 min. They were eventually affected by DAB (sigma-USA) then by secondary antibody. Subsequently, in each step, buffer phosphate was used. Positive control was blood and lymphatic vessels, squamous cell carcinoma. Primary antibody was removed and replaced with phosphate buffer which served as a negative control.

2.2. Method for studying the stained slides by CD34 in ameloblastoma and AOT

At first, microscopic slides were examined by 10x and 3 regions with maximum blood vessels density were considered; the mean blood vessels density in the 3 regions was recorded with magnification of 400 $\times$. The single endothelial cells stained with brown color and those that constituted vascular lumen in the form of cluster were considered. Blood vessels with muscle wall were excluded from the study.¹¹

2.3. Method of studying the stained slides by VEGFR3 in ameloblastoma and AOT

2.3.1. Lymphatic vessels

Microscopic slides were examined with magnification of 10 $\times$ and then, the regions with the highest lymphatic vessels (40x) were selected in five microscopic fields. It should be noted that lymphatic vessels were considered in the calculation by lumen formation.¹²

2.3.2. Odontogenic epithelial cells

If the membrane, cytoplasm of epithelial cells were colorable with VEGFR3, they were examined semi-quantitatively.

Negative: non-colorable.

+ Mild staining (10% color of the cells are colorable).

++ Medium staining (10–50% of the cells are colorable).

+++ Severe staining (more than 50% of the cells are colorable).¹³

3. Statistical analysis

The statistical data were entered into SPSS (20) and statistically analyzed by Mann-Whitney, ANOVA and T tests. Correlation coefficient was used to determine the relationship between eosinophilic density and lymphatic and blood vessels density in the odontogenic lesion.

The mean blood, lymphatic and eosinophils, vessels densities were recorded in the table in the form of Mean $\pm$ SD and P < 0.05 was considered significant.

4. Results

Demographics data of the samples are presented in Table 1.

4.1. Congo red staining and immunohistochemical results

The density of eosinophils in ameloblastoma was higher than that of AOT (see Table 2). In the central regions and adjacent to the odontogenic epithelium, both odontogenic lesions were more as compared to the peripheral areas. Their arrangement was focal (Figs. 1 and 2).

Blood vessel density of ameloblastoma was higher than that of AOT (P = 0/003) (Figs. 3 and 4). There was a direct and positive relation in the densities of eosinophils and blood vessel in ameloblastoma and AOT

Table 1

The mean of Age, Gender and Location, subtype of Ameloblastoma and AOT.

Lesion type	Lesion subtype	number	Location		Gender		Age Mean $\pm$ SD
			Mandible	Maxilla	Female	Male	
Ameloblastoma	10 follicular	20	16	4	8	12	40.15 $\pm$ 13.43
	5 Plexiform						
	3 Unisistic Mural						
	2 Unisistic luminal						
Adenomatoid Odontogenic Tumor	follicular	20	2	18	17	3	5.75 $\pm$ 18.69
P value			< 0.001		0.037		< 0.001

(P = 0.001, r = 0.539).

Although, the density of lymphatic vessels in ameloblastoma was higher than that of AOT, the difference was not statistically significant (P = 0/264). There was no positive relationship between eosinophils and lymphatic vessels densities in the two above mentioned odontogenic lesions (p = 0.288, R = 0.191) (Figs. 5 and 6) The average scores of odontogenic epithelium of ameloblastoma and AOT with VEGFR3 were respectively, 1.92 ± 0.5 and 1.62 ± 0.5 .

There was no statistically significant difference in the expression of the semi-quantitative VEGFR3 in epithelium of the two odontogenic lesions (P > 0.05). Expression of VEGFR3 was observed in peripheral cells (ameloblastic and central stellate reticulum) of the ameloblastoma. VEGFR3 seemed to uniformly stain duct-like cells of AOT.

5. Discussion

Different studies have always considered angiogenesis and lymphangiogenesis in relation to metastasis of cancers.¹⁴ In the present study, for first time, this relationship was compared with the clinical behavior of benign odontogenic tumors. In a text book, it was reported that ameloblastoma has aggressive behavior than AOT.²

Considering the results of this study, Expression of CD34, VEGFR3 and eosinophil densities appear to play a role in pathogenesis of ameloblastoma and AOT, it seems that angiogenesis and eosinophils density than lymphangiogenesis play a more significant role in the invasive behavior of ameloblastoma in comparison with the AOT. By secretion of angiogenic and anti-angiogenic factors, eosinophils can control vessels formation process in ameloblastoma.⁸ In this study, the increase in eosinophils density was associated with blood vessel density in ameloblastoma and AOT, but no relation was observed with lymphatic vessel density. In other words, eosinophils play no role in the generation of lymphatic vessels in ameloblastoma and AOT. Maybe, the generation of lymph vessels in ameloblastoma and AOT is done by other sources such as odontogenic epithelium and/or other inflammatory cells. Although, in this study, the density of lymphatic vessels in ameloblastoma is reported to be more than that of AOT, no significant difference was observed. Maybe, a more precise result could be obtained by increasing the sample size. Different markers, such as podoplanin and VEGFR3-Lyvel-Prox1, (D240), have been used for examination of lymphangiogenesis in various studies.¹⁵ In this study, we manipulated VEGFR3, because no study was investigated lymphangiogenesis in these selected odontogenic tumors with VEGFR3.

Sanchez-Romero et al. examined the relationship between CD34 and

Fig. 1. Positive congored staining in ameloblastoma (X40). **Fig. 2.** No congored staining in AOT (X40).

podoplanin expression in ameloblastic carcinoma, ameloblastoma and dental follicle. Furthermore, they studied the relationship between them with the invasive behavior of ameloblastic carcinoma. The expression of podoplanin in ameloblastoma was similar to teeth bud, but its expression in ameloblastic carcinoma showed a decrease. It seems

Table 2

The mean density of eosinophils, blood and lymph vessels in Ameloblastoma and AOT.

Lesion type	density of eosinophils		density of lymph vessels		density of blood vessels	
	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD
Ameloblastoma	3.2	$\pm$ 2.7	4.4	$\pm$ 2	14.9	$\pm$ 6.4
Adenomatoid Odontogenic Tumor	1.2	$\pm$ 0.7	3.6	$\pm$ 1.3	8.9	$\pm$ 3.4
P value	0.005*		0.264		0.003*

Fig. 3. Immunohistochemical staining with CD34 in ameloblastoma - Blood vessels stained with CD34 (x40).

Fig. 4. Immunohistochemical staining with CD34 in AOT -Blood vessels stained with CD34 (X40).

Fig. 5. Immunohistochemical staining with VEGFR3 in ameloblastoma-Lymph vessels stained with VEGFR3 (X40).

that podoplanin is not involved in the aggressive behavior of ameloblastic carcinoma, but increased blood vessel density is more effective in its invasive behavior.¹⁶

The results of the study conducted by Iwata et al.¹⁷ on renal cell carcinoma, lymphangiogenesis played a minimal key role in its improvement; but VEGFC, D is associated with histopathology characteristics and blood vessel density in the renal cell carcinoma. They used D2-40 and CD34 to evaluate immunohistochemistry.

In the present study, it is suggested that angiogenesis is more effective than lymphangiogenesis in invasive behavior of ameloblastoma as compared to AOT. It is consistent with results of the study conducted by Sanchez- Romero¹⁶ and Iwata et al.¹⁷ although, it is in contrast with the findings of Bgatova et al.¹⁸ who reported the importance of angiogenesis and lymphangiogenesis in tumor progression in melanoma. Several markers such as CD105, VEGF and CD34 are used to evaluate blood vessels by the immunohistochemistry methods. With CD34, old and new blood vessels are identified.^{11,19}

In review studies conducted, Alaeddini et al.,¹¹ Jamshidi et al.⁸ and Seifi et al.¹⁹ suggested the importance of blood vessel density (angiogenesis) in ameloblastoma in comparison with keratocyst and considered angiogenesis as the main mechanisms of invasive behavior of ameloblastoma. The results of the above studies are in accordance with the present one.

The main order of vessel formation and lymphangiogenesis is from odontogenic epithelium.^{15,19} In this study, the scores of epithelium staining in AOT and ameloblastoma were not different with VEGFR3. It means VEGFR3 has no major role in invasive behavior of ameloblastoma.

Based on the present study, expression of VEGFR3 in ameloblastic cells, stellate reticulum, peripheral, central cells of ameloblastoma and AOT were not different. It means ameloblastic cells, which have role reverse polarity in the invasive behavior of ameloblastoma, do not have much effect on lymphangiogenesis and invasiveness of ameloblastoma.

Ganvir et al. suggested that the higher expression of podoplanin in peripheral odontogenic epithelial cells of ameloblastoma are effective in tumoral invasive behavior,²⁰ which is in contrast to the obtained results on VEGFR3 in this study. It is believed that tumoral cells, stroma cells or inflammatory cells play a role in the generation of VEGF, and VEGFR3 has autocrine effect on the odontogenic epithelium and/or affect paracrine in the formation of new lymphatic vessels.^{21,22}

Since the expression of VEGFR3 in odontogenic tumors has not been investigated yet, in this study, the expression of VEGFR3 as a lymphatic marker was investigated.

Several studies have reported the role of podoplanin in lymphangiogenesis and in the recurrence and invasive behavior of keratocyst.¹⁵ Siar et al.²³ suggested the role of podoplanin in the recurrence and invasive behavior of ameloblastoma and induction of cell migration. However, according to the present study, there is no relationship between VEGFR3 expression and invasive behavior of ameloblastoma. In this study, an increase in eosinophils density consistent with blood vessels in ameloblastoma and AOT was observed. It seems that eosinophils and blood vessels in ameloblastoma and AOT are effective.

In this study, there was no difference in the expression of CD34 and VEGFR3 in follicular and plexiform of ameloblastoma, but in the study of Ganvir et al.²⁴ the expression of podoplanin associated with the cell type and its arrangement was reported in ameloblastoma.

There are several differences between the results of the current study and those of other studies. Perhaps, sample size, type of antibody used in evaluation of the blood vessels or lymphatic, and/or the method of assessing density of blood vessels, lymphatic vessels and eosinophils, affected the results.

By reviewing various articles, it appears that invasive treatments in ameloblastoma cause complications and problems to the patients.

Based on the results of this study, the hypothesis: anti - angiogenesis treatments in ameloblastomas may be more effective than *anti*-lymphangiogenetic therapies, can be suggested. Today, the VEGFR3 block

Fig. 6. Immunohistochemical staining with VEGFR3 in AOT- Lymph vessels stained with VEGFR3(X40).

(lymphatic vessels generation) infected by adenovirus gene is used for the treatment of lung, breast and stomach cancer.²⁵

6. Conclusion

The result suggest that tumor angiogenesis and eosinophil density may play a more effective role than lymphangiogenesis in local invasive behavior of ameloblastoma than AOT. Other techniques such as Western blot and real time PCR are necessary for our study results confirmation.

Declaration of competing interest

The authors have none to declare.

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