RESEARCH COMMUNICATION

Microvessel Density in Follicular Cysts, Keratocystic Odontogenic Tumours and Ameloblastomas

Safora Seifi*, Shahryar Shafaie, Siavash Ghadiri

Abstract

Purpose: Multicystic ameloblastoma is a benign epithelial odontogenic tumor that exhibits a more aggressive behavior than follicular cyst and keratocystic odontogenic tumor (KCOT). The aim of this study was to perform an immunohistochemical evaluation of the mean microvessel density (MVD) effect on clinical behavior of odontogenic lesions and to determine whether peritumoral or intratumoral MVD has a more prominent role in clinical behavior of odontogenic lesions. Methods: In a descriptive-analytic cross-sectional study, 45 paraffin blocks of mentioned lesions were selected and stained immunohistochemically with CD34. Mean MVD, peritumoral and intratumoral MVD for each odontogenic lesion was investigated and compared with each other. ANOVA and Kruskal Wallis were used for the statistical analysis of the results.Results:Mean MVD was 40.8±15.9, 25.3±5.4, and 9.4±3.52 in ameloblastoma, keratocystic odontogenic tumor , and follicular cyst, respectively. Mean MVD difference between the above mentioned lesions was statistically significant. (p<0.001) In all the odontogenic lesions, Intratumoral MVD was higher than peritumoral (pericystic) areas. (p=0.001) Conclusion: There was an increase of mean MVD in multicystic ameloblastoma in comparison to keratocystic odontogenic tumor and follicular cyst and it may be concluded as one of the main factors in multicystic ameloblastoma aggressive behavior. Intratumoral (intracystic MVD) has a more prominent role in growth and clinical behavior of mentioned odontogenic lesions. This supports the hypothesis that the early stages of growth and development of follicular cyst, KCOT and multicystic ameloblastoma may share some similarities regarding angiogenesis.

Keywords: Follicular cyst - keratocystic odontogenic tumor - ameloblastoma - immunohistochemistry - CD34

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Introduction

The dentigerous cyst (or follicular cyst) is one of the most common odontogenic cysts - thought to be of developmental origin – and is suggested to be caused by accumulation of exudate between the reduced enamel epithelium and the tooth crown. Enucleation of dentigerous cyst is the common treatment with very low recurrence and excellent prognosis (Zhang et al ., 2010). Odontogenic keratocyst is a common odontogenic cyst - thought to be of developmental origin – with aggressive behavior and high recurrence rate. The 2005 World Health Organization (WHO) classification of odontogenic tumors is the one of that introduced the term keratocystic odontogenic tumor. Orthokeratinized odontogenic keratocyst has a different clinical behavior than the parakeratotic odontogenic keratocyst and it is not associated with the inheriated nevoid basal cell carcinoma syndrome (Gorlin-Goltz syndrome).Therefore, orthokeratinized odontogenic cyst is not included in the 2005 WHO classification of odontogenic tumors(Gaitan-Cepeda et al.,2010). Keratocystic odontogenic tumors are sometimes related to nevoid basal cell carcinoma syndrome, which is a rare inheritance disorder caused by mutations in the PTCH gene on chromosome 9 causing multiple odontogenic keratocyst of the jaws, basal cell carcinoma (BCC) of the skin, and vertebral anomalies. The severity of the skin BCC determines the prognosis(Sasaki et al . ,2010). High epithelial proliferation and increase of matrix metalloproteinases are known to be among growth mechanisms. It's treatment is by enucleation along with curettage has been reported to have 5-62% recurrence rate (Kolar et al .,2006).

Multicystic ameloblastoma is considered as the most common benign epithelial odontogenic tumors. It is a locally invasive tumor that has a tendency to continually grow and invade the surrounding tissues. Malignant changes in multicystic ameloblastoma have been reported in less than 5% of cases. Some cases of metastasis have also been reported. Marginal resection (MR) is the main treatment practice. The probability of recurrence cases after curettage is high (Gomes et al., 2010 ; Van Dam et al.,2010).

It seems that little attention has been paid to the connective tissue of odontogenic cysts and stroma of odontogenic tumors and some researches have been on epithelial proliferation and apoptotic factors (Kichi et al.,2005) . Blood supply is a essential factor for the growth

*Oral and Maxillofacial Pathology , School of Dentistry, Babol University of Medical Sciences, Babol, Iran *For correspondence : asim.sf_seify@yahoo.com*

of odontogenic epithelium. Because there is no vascular system in the epithelium, apoptosis will happen if the connective tissue does not provide the necessary blood supply (Karamysheva., 2008). Myofibroblasts, blood vessels, and inflammatory cells are present in the tumoral stroma. The invasion and metastasis of tumors requires the presence of myofibroblasts and blood vessels which increases during tumorigenesis (Kademani et al., 2009; Seifi et al., 2010).

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels and, like cancer, is a complex multi-stage process including degradation of extracellular matrix, proliferation and migration of endothelial cells, capillary differentiation and anastomosis (Kumamoto et al., 2002). Some research has been done on angiogenesis in melanoma (Mahabeleshwar et al.,2007) , squamous cell carcinoma (Margaritescu et al ., 2010) , salivary gland tumors (Soares et al.,2009). Different markers like CD34, CD31, CD105 and antibodies like VEGF, bFGF, Tek2 are used to measure microvessel density in each microscopic field. In comparison to antibodies, the markers are more practical for the measurement of microvessel density (Kademani et al.,2009).

CD34 staining is stronger with lower error rate in comparison to CD31. Sialomucin CD34 is a cell surface 110-120 KD monomeric glycoprotein and is a pan-endothelial marker of endothelial cells and normally shows stronger staining with endothelial cells. However, some researches believe that CD34 cannot be used to distinguish between the new formed blood vessels and the old host ones but CD34 has main role in evaluation of microvessel density in tumors (Lanza et al., 2001). Until now, few studies have reported on the role of angiogenesis in odontogenic lesions and little has been known on the role of angiogenesis in odontogenic cysts and tumors. The current study aimed to perform an immunohistochemical evaluation of the mean microvessel density effect on clinical behavior of odontogenic lesions and to determine whether peritumoral or intratumoral microvessel density has a more prominent role in clinical behavior of odontogenic lesions.

Materials and Methods

The study protocol was approved by our Institutional Ethics Committee. In a retrospective cross-sectional study, first medical records of patients existed in the archive of the school of dentistry between 2003 and 2009, and the records existed in Shahid Beheshti Hospital between 1991 and 2009 were reviewed. The samples diagnosed with odontogenic lesions including dentigerous cyst (follicular), keratocystic odontogenic tumor, and multicystic ameloblastoma were selected.

Clinical information including age, sex, and the location of the lesion was extracted from patients' files and recorded in tables. Related paraffin blocks were then selected. To confirm the diagnosis and inclusion of the samples in the study, first 5-micron sections were prepared and were stained using hematoxylin-eosin staining protocol. The diagnosis was then confirmed by two pathologists. Sections with enough tissue with suitable fixation were selected and those with inflammation and hemorrhage and insufficient tissue and incisional bipsy were excluded from the study. Also, all the orthokeratotic odontogenic keratocyst and keratocystic odontogenic tumor cases related to Gorlin–Goltz-syndrome were excluded from the study.

Of all the 45 paraffin blocks, 15 samples of each type were selected that based on the Neville et al (2009) definition had the histopathological characteristics of follicular cyst, keratocystic odontogenic tumor, and multicystic ameloblastoma. The two common forms of multicystic ameloblastoma (plexiform and follicular) were selected for the study.

For the immunohistochemical staining, four-micron sections were prepared from each paraffin block and were deparaffinized in xylene and dehydrated in graded alcohol series. To block the internal peroxidase activity they were placed in hydrogen peroxide (3%) in phosphate buffer. Antigen retrieval was done in a microwave oven (Panasonic 1380W) for 10 minutes , under the pressure of 2 atmosphere in 120 degree centigrade. Anti CD34 (QBend 10, A/S, Glostrup, DAKO, Denmark) was used as the primary antibody for 30 minutes and was incubated in a moist chamber at room temperature (24h) with a working dilution 1:50 . followed by the application of secondary antibody (for 15 minutes), DAB (to produce brown staining), and Meyer's hematoxylin (for background staining). The samples were placed in phosphate buffer saline (PBS) after each mentioned step. Squamous cell carcinoma was the positive control. The negative control was obtained by the replacement of primary antibody with PBS. Also, 4 cases of normal oral mucosa around hyperkeratosis were selected as internal positive control.
Assessment of immunohistochemistry stained sections:
In brief, immunohistochemical evaluation were performed based on Weidner et al. method (Weidner et al.,1995). All samples were observed at 10-time-magnification under the light microscope (Olympus,BX41, Tokyo, Optical, Japan).Three areas with the highest number of blood vessels (hot spots) were selected. The number of blood vessels in each field was then studied at 40-time-magnification.The area of each field was almost 0.2 mm2. The blood vessels density was recorded as Mean ± SD. Those endothelial cells colored with brown CD34 (CD34-positive) that formed a cluster of endothelial cells with a lumen were considered as blood vessels. Single CD34-positive endothelial cells were also included in the count.Blood vessel with muscle wall was excluded.The histomorphometric analysis of blood vessels was carried out by Motic plus2 software was attached with Olympus BX41 microscope.

Intratumoral (intracystic) microvessel density represented hot spots that were in the center of the tumor (cyst) in an area of $2mm^2$ under basal lamina of epithelium. Peritumoral microvessel density in multicystic ameloblastoma represented hot spots located at periphery tissue within $2mm^2$ of tumor islands from invasive front (Wang et al., 2010).Peritumoral (pericystic MVD) in follicular cyst and keratocystic odontogenic tumor included hot spots located at periphery connective tissue,

Table 1. Demographic and Vessel Density Data

Lesion	Number	Sex (F)	Sex (M)	Average Age	Location Mandible	Location Maxilla	Peritumoral MVD	Intratumoral MVD	Mean MVD
Follicular cyst	15	7	8	22.3+2.2	13	2	8.1+1.5	7.7+1.6	4.9+3.5
Keratocystic odontogenic tumor	15	3	12	28.2+3.4	15	0	7.3+1.6		
Multicystic ameloblastoma	15	7	8	36.4+3.8	14	1			

MVD, microvessel density; *,**

almost 2mm2 Space from its end zones,almost in near area of bone trabecules. All the immunohistochemically stained slides were reviewed by two pathologists and both agreed on the number of counted vessels.

Kruskal Wallis test was for compare means in groups; and the Mann-Whitney was used to compare multiple pairs. The statistical significance level set at 0.05.

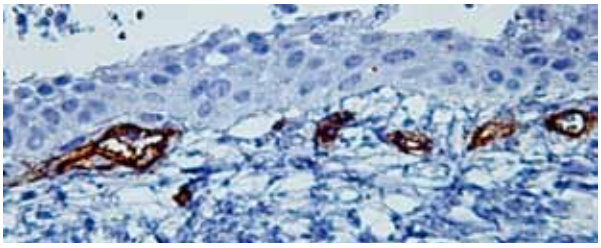

Figure 1: Immunohistochemical staining with CD34 marker in follicular cyst (X40) Staining of blood vessels adjacent to odontogenic epithelium. (Intracystic area)

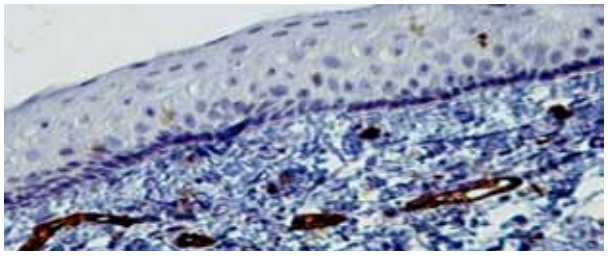

Figure 2: Immunohistochemical staining with CD34 marker in Keratocystic odontogenic tumor (X40 Staining of blood vessels adjacent to odontogenic epithelium. (Intratumoral area)

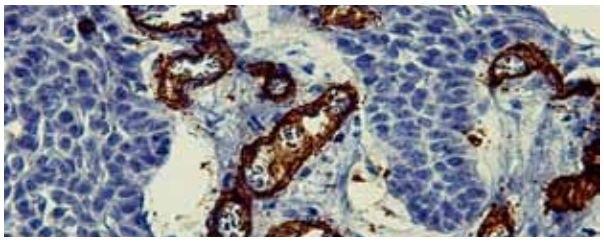

Figure 3: Immunohistochemical staining with CD34 marker in follicular ameloblastoma(X40) Staining of blood vessels with CD34 marker (Intratumoral MVD)

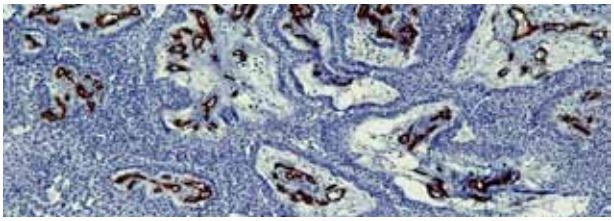

Figure 4: Immunohistochemical staining with CD34 marker in plexiform ameloblastoma(X10) Staining of blood vessels with CD34 marker

Results

Demographic results have been summarized in Table 1. Positive staining was immunohistochemical brown cytoplasmic color in vascular endothelial cells. Mean MVD in multicystic ameloblastoma, keratocystic odontogenic tumor , and follicular cyst was 40.8±15.9, 25.3±5.4, and 9.4±3.52, respectively.Mean MVD difference in follicular cysts, keratocystic odontogenic tumors and multicystic ameloblastoma was statistically significant. ($p<0.001$) Mean MVD in keratocystic odontogenic tumor was higher than follicular cyst. ($p<0.001$) (Figures 1-4).

Blood vessels in high numbers and small size were abundant in multicystic ameloblastoma. Anastomosis was seen in some and it was mostly observed around tumor islands of multicystic ameloblastoma. In follicular cysts and keratocystic odontogenic tumors, fewer blood vessels that were relatively bigger were observed especially in the sub-epithelial area of odontogenic epithelium. Immunohistochemical results have been summarized in table2.

In plexiform ameloblastoma (n=7 IT SAYS 15 IN THE TABLE!!), mean MVD was 27.5±7.6 and in follicular ameloblastoma (n=8) it was 52.4±11.1. Mean MVD of follicular type was higher than plexiform. ($p<0.001$)

Blood vessels in high numbers and smaller size were abundant in follicular ameloblastoma and were more dilated and more distributed in less numbers in plexiform ameloblastoma.

Discussion

In the current study microvessel density in multicystic ameloblastoma was higher than keratocystic odontogenic tumor and follicular cyst which in a form suggests a higher angiogenesis in odontogenic tumor lesions with higher aggressive behavior in comparison to odontogenic cyst. Angiogenesis is a complicated process happening both in physiologic and pathologic conditions(Kumamoto et al., 2002).

Angiogenesis acts like a double-edged sword, valuable in normal physiologic conditions but uncontrollable and invasive in neoplastic and inflammatory conditions(Kumar et al.,2005). Epithelial tissue stroma is of the major supporting factors. Any changes in epithelium with cause some changes in the stroma. The changes in the host stromal tissue are because of unbalanced secretion of cytokinins and the changes in the number of blood vessels (Seifi et al.,2010 ; Kademani et al., 2009). The blood vessels in stroma are one of the essential factors of epithelial growth (Kumar et al.,2005). The growth, aggressive behavior, and metastasis of a tumor need

nutritional substances and oxygen that is provided by blood vessels. When the size of a tumor reaches beyond 2 cm, the tumor will not be able to grow further. Tumor angiogenesis happens through the host's blood vessels. Tumor cells secret metaloproteinase causing degradation of basement membrane of the host's blood vessels, proliferation of endothelial cells, their migration, and formation of new vessel lumen (Kademani et al., 2000 ; Inda et al.,2007).

Different studies have reported on matrix metalloproteinases expression and it's inducers in connective tissue of follicular cyst, stroma of keratocystic odontogenic tumor and multicystic ameloblastoma. However, it has been reported to have a higher frequency in multicystic ameloblastoma than keratocystic odontogenic tumor and dentigerous cyst. Meanwhile, some previous studies have reported a positive relationship between matrix metalloproteinases inducers and the vascular density. Because the presence of matrix metalloproteinases is essential for angiogenesis, it's higher expression in multicystic ameloblastoma may be involved in higher rate of angiogenesis and it's higher clinical invasive behavior. (Ali .,2008 ; Jiang et al.,2008)

The growth mechanism of keratocyst is different from follicular cyst. Some researches suggest that increase in epithelial anti-apoptotic agents and increase in stromal metaloproteinases of connective tissue affect the keratocysts growth. It seems that one of the effective factors increasing angiogenesis in keratocystic odontogenic tumors in comparison to follicular cysts is the increase of stromal metaloproteinases of connective tissue as the affects the keratocystic odontogenic tumor angiogenesis(Geitan – Cepeda et al., 2010,Kolar et al., 2006).

In the current study, microvessel density in keratocystic odontogenic tumor was higher than follicular cyst which is a kind of explanation for higher recurrence rate and aggressive behavior of keratocystic odontogenic tumor in comparison to follicular cyst. Also it is suggested higher need tumoral tissue to nutritional substances and oxygen than follicular cyst.

Follicular cyst has excellent prognosis and little recurrence after treatment(Zhang et al.,2010). The behavior of this cyst can be explained by it's lower rate of angiogenesis in connective tissue in comparison to keratocystic odontogenic tumor and multicystic ameloblastoma.

Using CD34 and immunohistochemical methods, Alaeddini et al. in 2009 studied and compared the angiogenesis in odontogenic keratocyst (20 cases), dentigerous cyst (14 cases), and ameloblastoma (20 cases). Mean microvessel density in ameloblastoma was higher than keratocystic odontogenic tumor and follicular cyst(Alaeddini et al.,2009). The results of the mentioned study are somehow in line with the results of Alaeddini's et al. study. They did not compare the Intatumoral and peritumoral microvessel density of odontogenic lesions.

Gadbil et al. studied the relationship between proliferative activity in epithelial cells keratocystic odontogenic tumor and follicular cyst and normal oral mucosa with angiogenesis using CD 105 marker. They reported higher CD105 expression in keratocystic odontogenic tumor in comparison to dentigerous cysts and concluded that keratocystic odontogenic tumor stroma was involved in the observed neoplastic behavior (Gadbil et al .,2010) . Gadbil's et al study are same in line with our research result.

The distribution of blood vessels across all the zones of an odontogenic lesion is not the same. In the mentioned study, Intratumoral microvessel density of multicystic ameloblastoma showed the highest rate of angiogenesis. The angiogenesis rate was low in peritumoral area of multicystic ameloblastoma. It seems that epithelium of the odontogenic lesions has a major role in induction of angiogenesis in the connective tissue and leads the changes direction in the connective tissue. Although based on the recurrence and aggressive behavior, it is expected to see higher angiogenesis in peritumoral zones of neoplastic lesions (Dunstan et al., 1997), in this study, in comparison to peritumoral zones , we observed higher microvessel density in intratumoral areas and areas adjacent to odontogenic epithelium. This is a sign of multicystic ameloblastoma tumoral cells activity inducing angiogenesis. It seems that accumulation of blood vessels around odontogenic epithelium in order to provide oxygen and nutritional substances is critically important for the growth of odontogenic cyst and tumors.

Although the growth mechanism of follicular cyst is different from keratocystic odontogenic tumor which is through an increase in osmotic pressure (Neville et al.,2009), it seems that angiogenesis has the primary role in development and growth of follicular cysts. Without the presence of blood vessels in intracystic area the epithelium of the cysts will not be able to grow. In other words, angiogenesis is of primary mechanisms before the increase in osmotic pressure and is effective in the growth and development of follicular cyst. Considering the increase of intratumoral microvessel density in keratocystic odontogenic tumor in comparison to peritumoral microvessel density, it seems that follicular cyst and keratocystic odontogenic tumor have similar early stages of growth which is mostly dependent on blood vessels of intratumoral. However, the vascular density and vessel area of these odontogenic lesions seems to be different.

So researches believe that the microvessel density can be used as a practical factor to predict the relapse or invasiveness and metastasis of a tumor(Kim et al.,2006). However, some others think that the number of blood vessels in peritumoral and intratumoral zones is the same and they do not believe that it can be used for the prediction of aggressive behavior and recurrence(Margartescue et al.,2010).The results of this study are in accordance to the first group.

Chen et al. used immunohistochemical methods to study the expression of CD34, VEGF, and INOS in 35 cases of ameloblastoma (primary tumor, recurrence, and malignant) and keratocyst. They reported an increase in microvessel density from keratocyst to recurrent ameloblastoma to malignant ameloblastoma. They also mentioned microvessel density that was higher in ameloblastoma when compared to keratocyst(Chen et al.,2009). Regarding higher vascular density in

multicystic ameloblastoma in comparison to keratocystic odontogenic tumor, the results of the mentioned study is similar to the present study.

Although the number of blood vessels is higher in multicystic ameloblastoma, they are smaller in size and anastomosis was seen, in keratocystic odontogenic tumors the number of blood vessels is less and in follicular cysts the size of blood vessels seems bigger. Nevertheless, it is better to use histomorphometry method to measure the vessel area and diameter of blood vessels in order to evaluate the role of vessel area effect in aggressive behavior of odontogenic lesions. However, Gadbil et al. reported a bigger vessel area in keratocystic odontogenic tumor in comparison to dentigerous cysts(Gadbil et al.,2010).

When selecting the paraffin blocks of follicular cyst, keratocystic odontogenic tumors, and multicystic ameloblastoma, the samples with severe inflammation were excluded from the study because inflammation can affect the microvessel density. However, in multicystic ameloblastoma there were areas of stromal inflammation with focal increase of microvessel density. This can suggest that inflammatory cells need nutritional substances for their activities.

The correlation between inflammation and angiogenesis in radicular cysts using VEGF antibody was studied by Graziani et al.. They reported an increase in microvessel density with an increase in inflammation[Graziani et al.,2006].

Although some researchers do not think histopathological type of multicystic ameloblastoma is effective in its aggressive behavior and suggested similar prognosis for follicular and plexiform type (Neville et al., 2009), in the current study, though the number of follicular and plexiform samples were few, the decrease of microvessel density in plexiform type in comparison to follicular type was noticeable. This may suggest a more aggressive behavior for follicular type in comparison to plexiform type. In other words, the expression of CD34 and mean vascular density may be a determining factor in prognosis and prediction of the odontogenic tumor progress.

Kumamoto et al. studied the angiogenesis in multicystic ameloblastomas and reported that no microvessel density difference was observed between the histopathological form of follicular type and the plexiform type (Kumamoto et al.,2002).However, in our study as well as the study of Tete et al., the microvessel density was higher in follicular type in comparison to plexiform type (Tete et al.,2005). Sample volume and the differences in methodology can be the reasons of the observed differences between the current study and the Kumamoto's et al study (Kumamoto et al.,2002). Smaller blood vessels in size and higher in numbers were observed in follicular type in comparison to plexiform type. However, the difference was not statistically significant. They reported that basal cell ameloblastoma had the lowest number of blood vessels.

There are some limitations in discussing the results of the current study due to the few numbers of studies on angiogenesis in odontogenic lesions. However, the majority of studies have emphasized on the role of angiogenesis as an important and prominent factor in aggressive behavior of odontogenic lesions. Histomorphometry and ultrastructure study by el-Labban et al.(el-labban et al.,1990) did not report any difference in microvessel density between dentigerous cysts and odontogenic keratocyst. This could be due to the different techniques that were used for the evaluation of microvessel density.

Immunohistochemical expression of vascular endothelial growth factor (VEGF) in dentigerous cyst and parakeratotic odontogenic keratocyst and orthokeratic odontogenic keratocyst was studied by Rubini et al. in 2010. VEGF expression in keratocyst was higher than dentigerous cyst and more than 50% of endothelial cells in 88% of parakeratinized odontogenic keratocyst cases and 68% of orthokeratinized type of keratocyst , positive VEGF staining were observed. They reported angiogenesis as the active mechanism of aggressive behavior of parakeratinized keratocyst in comparison to other odontogenic cysts (Rubini et al.,2010) which, from some aspects, is in line with the results of the current study.

In immunohistochemical evaluation using CD31 in radicular cyst and odontogenic keratocyst, Tete et al. observed the positive staining in all the cases and reported that angiogenesis has a primary role in the development of cysts of the jaw. .[Tete et al.,2005] In the mentioned study the increase in intracystic microvessel density in comparison to pericystic area in follicular cyst is an expression of active role of intracystic microvessel density cyst in the growth and development of the mentioned cyst.

In summary, it seems that mean microvessel density increases from follicular cyst to keratocystic odontogenic tumor and multicystic ameloblastoma. This reflects the major role of angiogenesis in aggressive behavior of odontogenic lesions. For the aggressive behavior of multicystic ameloblastoma in comparison to odontogenic cysts Angiogenesis in intratumoral zone has a more prominent role than angiogenesis in peritumoral zone of odontogenic lesions. This supports the hypothesis that the early stages of primary growth and development of follicular cyst, KCOT and multicystic ameloblastoma may share some similarities regarding angiogenesis.

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