

Original Article

The role of vascular endothelial growth factor in proliferation of odontogenic cysts and tumors: An immunohistochemical study

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) is capable of initiating angiogenesis in blood vessels and may act as mitogenic agent for epithelium of odontogenic cysts and tumors. This study was conducted to evaluate the role of epithelial VEGF expression in odontogenic cysts and ameloblastoma and its correlation with argyrophilic nucleolar organizer region counts to assess its role in their biological behavior.

Materials and Methods: In this retrospective cross-sectional study, 45 histologically confirmed cases, 15 cases of each of keratocystic odontogenic tumors (KCOTs), dentigerous cysts, and ameloblastomas were examined for immunohistochemical expression for epithelial VEGF, and argyrophilic nucleolar organizer regions (AgNORs) (used as secondary marker in this study) staining was done for comparing the proliferative capacity with VEGF.

Results: KCOT shows mild expression within the basal layers and strong expression in the suprabasal layer whereas, in dentigerous cysts, a majority showed no VEGF expression whereas ameloblastomas showed strong expression in all cases by stellate reticulum-like cells at the center of the follicles and suprabasal layers of epithelium. The results of AgNOR counts were higher in KCOTs as compared to ameloblastoma and least in dentigerous cysts.

Conclusion: VEGF expression by the epithelium of odontogenic cysts and tumors may play a role in epithelial proliferation via autocrine mechanism as reflected by increased AgNOR counts. The angiogenic activity via paracrine pathway may be responsible for the difference in growth rate and neoplastic behavior of the lesions.

Key Words: Argyrophilic nucleolar organizer region associated proteins, odontogenic tumor, vascular endothelial growth factor

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INTRODUCTION

Cysts and tumors constitute an important aspect of oral and maxillofacial pathology.^[1] Vascular endothelial growth factor (VEGF) is a heparin binding, dimeric glycoprotein with a selective mitogenic effect on the vascular endothelial cells and is capable of initiating

angiogenesis in blood vessels.^[2,3] In previous studies, VEGF had been used as tumor marker in stroma which marks lymphocytes, macrophages, and fibroblasts from endothelial cells.^[4,5] Only a few studies in cysts and tumors have been done to

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evaluate epithelial expression of VEGF. The results of these studies showed epithelial VEGF expression as a mitogenic agent.^[6] Hence, in the present study, we used VEGF to evaluate its expressions in epithelium of these lesions and know whether its expression may in some way be responsible for the higher proliferative capacity of epithelial cells in keratocystic odontogenic tumor (KCOT) and ameloblastoma and whether it act as a mitogenic agent for epithelium of odontogenic cysts and tumors.^[2,4,5] Argyrophilic nucleolar organizer region (AgNOR) is used to study cell proliferation in various types of tumors.^[7,8]

The rationale of the current study was to compare the expression of VEGF in dentigerous cyst, KCOT, and ameloblastoma and the correlation with their proliferative potential, using AgNOR counts, to assess the role of VEGF in their biological behavior.

MATERIALS AND METHODS

The present retrospective cross-sectional study was carried out on biopsy tissues obtained from the archives of the Department of Oral and Maxillofacial Pathology. The study sample included 45 cases of odontogenic cysts and tumors, 15 cases of each of KCOTs, dentigerous cysts, and ameloblastomas. The diagnoses were reviewed using routine hematoxylin and eosin stained sections. Inclusion criteria were all subtypes of ameloblastomas, dentigerous cyst, and KCOT and were taken from both male and female patients of age between 16 and 60 years without any inflammation. Exclusion criteria were patients having any other oral lesions along with cysts and tumors, any systemic diseases, pregnancy, and tobacco habits.

Immunohistochemical staining for vascular endothelial growth factor

The detection of VEGF was performed using primary antibody (Biogenex Super sensitive Polymer Horse Radish Peroxide immunohistochemical [IHC] Detection Kit [BioGenex Life Sciences Pvt. Ltd., Hyderabad, Andhra Pradesh, India] and secondary antibody of Leica NovoLink Polymer Detection System [Newcastle upon Tyne NE, United Kingdom]). Positive and negative controls were run with each batch of staining. Positive control consisted of paraffin-embedded sections of oral squamous cell carcinoma (OSCC) with known antigenic reactivity to VEGF.

Immunohistochemistry staining procedure

Formalin-fixed paraffin-embedded tissues were sectioned at 3 μ m and mounted on poly-L-lysine

coating slides and were incubated in a preheated incubator at 65°C for 1 h. Sections were dewaxed in xylene (two changes) and then rehydrated in graded alcohol. The sections were kept in a coupling jar filled with citrate acid buffer (pH 7.2) and placed in a microwave oven and were given two cycles at high (80°C) mode, one cycle at medium high (60°C) and one cycle at low (40°C), each lasting for 5 min; all these steps were done according to the protocol given in Leica NovoLink Polymer Detection System (Newcastle upon Tyne NE, United Kingdom). The sections were then allowed to cool to room temperature. Endogenous peroxidase activity was blocked by incubating the sections with peroxide block (3% hydrogen peroxide) for 12 min. Sections were treated with power block and incubated with anti-VEGF rabbit polyclonal antibody (BioGenex Life Sciences Pvt. Ltd., Hyderabad, Andhra Pradesh, India) for 1 h in a humidifying chamber at 37°C. After buffer wash, the sections were incubated with postprimary for 30 min. After rinsing in buffer, sections were then incubated with NovoLink Polymer (anti-mouse/rabbit IgG-poly-HRP) secondary antibody. The sections were incubated with 3,3-diaminobenzidine tetrahydrochloride chromogen. Subsequently, after rinsing in distilled water, it is counterstained with hematoxylin (Harris hematoxylin given in the kit).

Argyrophilic nucleolar organizer region staining solutions

Two solutions were taken, Solution A and B.

Solution A

Silver nitrate (Fisher Scientific Qualigen, Waltham, Massachusetts) - 50 g.

Deionized water - 100 ml.

Solution B

Gelatin powder - 2 g.

Formic acid - 1 ml.

Deionized water - 100 ml.

Each time, the final working solution was freshly prepared by mixing one volume of Solution A and two volumes of Solution B.

Staining procedure

The slides were subjected to AgNOR staining according to the method of Ploton *et al.*^[9] The slides were progressively rehydrated through descending grades of alcohol and dewaxed in xylene. Finally, the slides were washed with deionized water. Excess water was shaken off from the slides and the freshly

prepared working solution was poured over the slides. These were then placed in the incubator at 37°C for 30 min. After staining, the slides were washed in deionized water, followed by sequential dehydration in ascending grades of alcohol. The slides were cleaned in xylene and mounted in synthetic medium (DPX).

Assessment of immunohistochemical expression of vascular endothelial growth factor

Qualitative assessment

Following the IHC staining, all the stained sections were analyzed under bright field microscope (Olympus BX-51, Japan). VEGF expression was assessed in the epithelial cells of dentigerous cysts, KCOTs, and ameloblastoma. Qualitative assessment of VEGF expression was performed based on the localization and intensity of staining and was graded into four grades such as no expression (0), mild expression (1), moderately strong expression (2), and strong expression (3). The expression of VEGF in positively stained epithelial cells was graded according to criteria given by Mitrou *et al.*^[2] [Table 1].

Criteria for each grade

According to Mitrou *et al.*, for dentigerous cysts and KCOT, the epithelial lining was divided into two zones, i.e., basal cells and suprabasal cells.^[2] In ameloblastoma, the epithelium was divided into two zones, i.e., basal/peripheral ameloblast-like cells and suprabasal/central stellate reticulum-like cells.^[2]

Quantitative assessment

For quantitative analysis, all the sections were first examined at ×100 magnification and three fields of strongest VEGF expression were selected. These selected fields were then photographed at ×200 magnification (Olympus BX-51, Japan). The photographs were analyzed using Image Pro Express version 6.0 (Media cybernetics manufacturing, 780 Commonwealth Drive, Warrendale, PA 15086, USA) for windows for both VEGF and AgNOR. In this study, first, the VEGF-positive epithelial cells

were counted in the photograph and then the total number of epithelial cells/tumor cells in cysts and ameloblastomas were counted in each photograph irrespective of the level (i.e., basal/peripheral or suprabasal/central) as well as staining intensity. The percentage of VEGF-positive cells was calculated and was considered as “VEGF labeling index (LI).” The results of both qualitative and quantitative analysis were made after interobserver observations.

AgNOR staining – For this staining, again separate slides, 45 slides, irrespective of VEGF (IHC procedure), of dentigerous cysts, KCOTs and ameloblastomas were taken. The slides were subjected to AgNOR (Fisher Scientific, New Hampshire) staining according to the method of Ploton *et al.*^[9] All sections were examined under ×1000 magnifications in oil immersion using light microscope, and AgNOR dots were counted in 100 randomly selected cells using point counting tool from the basal and parabasal layers.

Statistics

The Chi-square test was done in all three groups along with, *post hoc* analysis for intergroup comparison and one-way ANOVA between VEGF and AgNOR counts. $P < 0.001$ was considered statistically significant.

RESULTS

VEGF expression was seen both in the epithelial and the connective tissue components. VEGF was strongly expressed in the cytoplasm of vascular endothelial cells and lymphocytes, hence used as internal positive control.

In dentigerous cysts, the majority of the cases showed no epithelial VEGF expression. In basal layer, 11 cases showed no expression whereas four cases showed mild expression. In suprabasal layer, nine cases showed no expression whereas five cases showed mild expression [Graph 1 and Figure 1a].

In KCOT, VEGF expression was seen in all 15 cases. The pattern of expression was uniform with all cases showing mild expression within the basal layers and 14 of 15 cases showing strong expression, and one case showed moderately strong expression in the suprabasal layer [Graph 2 and Figure 1b].

VEGF expression was observed in all 15 cases of ameloblastoma. VEGF was strongly expressed by the stellate reticulum-like cells at the center of the follicles or strands and suprabasal layers of lining epithelium in cystic cases (unicystic ameloblastomas).

Table 1: Grading criteria given by Mitrou *et al.*

Grade	Type of expression
No expression (-)	Complete absence of staining
Weak expression (+)	Light/faint cytoplasmic staining or sporadic positive cells
Moderately strong expression (++)	Moderately intense cytoplasmic staining, diffusely present throughout the epithelium/moderately intense to intense cytoplasmic staining at multiple foci
Strong expression (+++)	Intense cytoplasmic staining throughout the epithelium

On the peripheral, ameloblast-like cells showed mild VEGF positivity in all cases [Graph 3 and Figure 1c].

In dentigerous cyst, the quantitative analysis of VEGF expression (VEGF LI) ranged from 0% to 93.4% (mean 27.16%). In KCOT, LI ranged from 80% to 100% positive cells (mean 94.32%). LI in ameloblastoma ranged from 83.7% to 100% (mean 95.37%). LI was found to be statistically significant ($P < 0.001$) [Table 2 and Figure 2a].

Post hoc analysis for intergroup comparison showed that LI was significantly lower in dentigerous cyst

as compared to KCOT ($P < 0.001$) as well as ameloblastoma ($P < 0.001$) [Table 3 and Graph 4]; however, there was no significant statistical difference between KCOT and ameloblastoma ($P = 0.992$).

Argyrophilic nucleolar organizer region counts in dentigerous cyst, keratocystic odontogenic tumor, and ameloblastoma

In dentigerous cyst, AgNOR count ranged from 1.0 to 1.25 (mean 1.11) AgNOR dots/nuclei. In KCOT, AgNOR count ranged from 1.32 to 2.92 (mean 2.39) AgNOR dots/nuclei. In ameloblastoma, AgNOR count ranged from 1.43 to 2.84 (mean 1.86) AgNOR dots [Table 2 and Graph 5]. The mean AgNOR counts between the study groups were compared using one-way ANOVA test and the differences were found to be significant ($P < 0.001$). The AgNOR count of KCOT was found to be higher than ameloblastoma, and this difference was also statistically significant ($P < 0.001$). Our study showed higher VEGF expression in KCOTs and ameloblastoma as compared

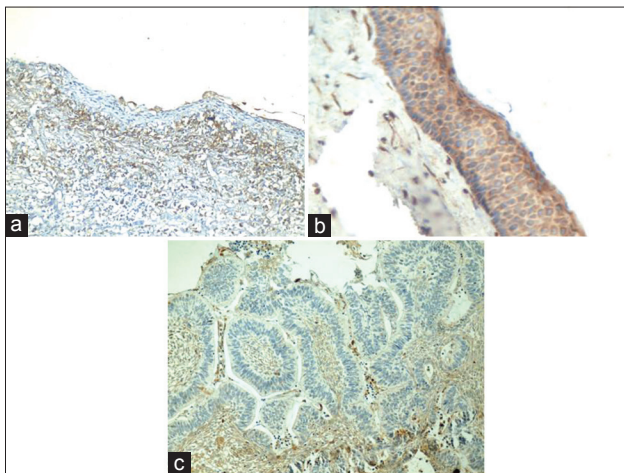
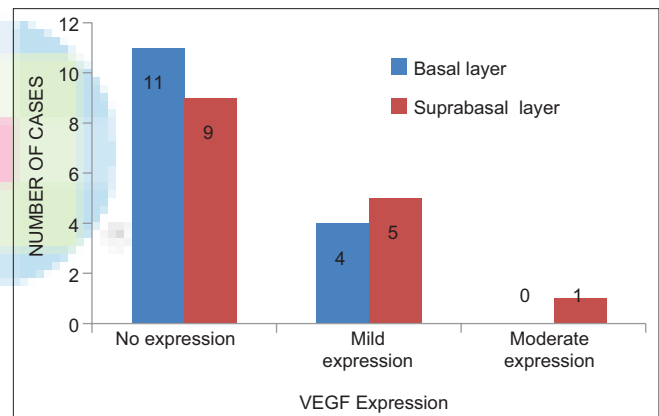


Figure 1: (a) Photomicrograph showing strong vascular endothelial growth factor expression in stellate reticulum-like cells in ameloblastoma (×200). (b) Photomicrograph showing lack of epithelial vascular endothelial growth factor expression in dentigerous cysts. Note: The stromal cells show positive vascular endothelial growth factor expression (×200). (c) Photomicrograph showing strong vascular endothelial growth factor expression in keratocystic odontogenic tumor (×200).



Graph 1: Vascular endothelial growth factor expression in dentigerous cysts.

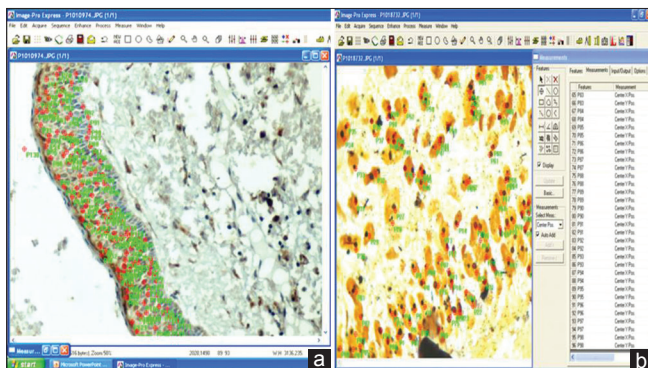
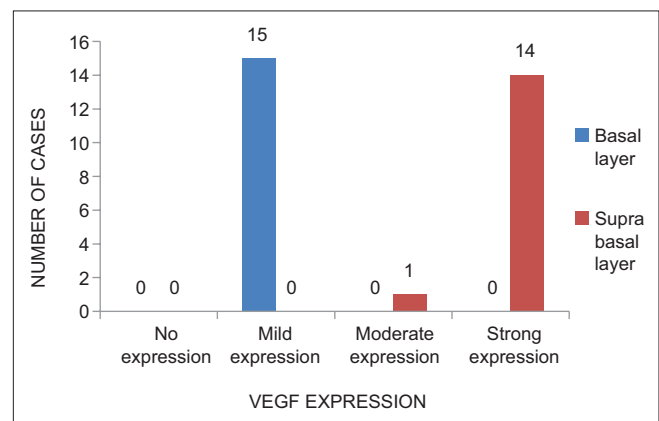


Figure 2: (a) Photomicrograph showing counting procedure of vascular endothelial growth factor labeling index in basal and suprabasal layers of epithelium using Image Pro Express (×200). (b) Photomicrograph showing counting argyrophilic nucleolar organizer region dots using Image Pro Express (×1000).



Graph 2: Vascular endothelial growth factor expression in keratocystic odontogenic tumor.

Table 2: Comparison of vascular endothelial growth factor labeling index and argyrophilic nucleolar organizer region counts between study groups

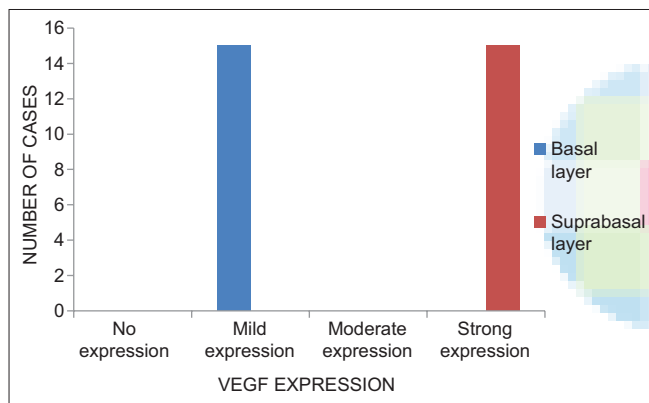
Groups	n	Mean		SD		Statistics/mean squares		df2 (welch)/ F (ANOVA)		P	
		VEGF LI	AgNOR	VEGF LI	AgNOR	VEGF LI	AgNOR	VEGF LI	AgNOR	VEGF LI	AgNOR
Dentigerous cyst	15	0.2716	1.112	0.39576	0.091745	17.23	48.357	24.52	19.71	<0.001	<0.001
KCOT	15	0.9432	2.306	0.06147	0.535666						
Ameloblastoma	15	0.95366	1.884	0.04755	0.537452						
Total	45	0.72282	1.767	0.36813	0.660376						

SD: Standard deviation; VEGF: Vascular endothelial growth factor; AgNOR: Argyrophilic nucleolar organizer regions; KCOT: Keratocystic odontogenic tumor; LI: Labeling index

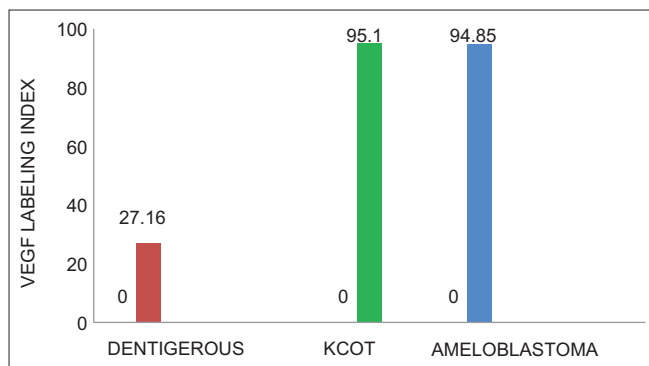
Table 3: Post hoc analysis for intergroup comparison of vascular endothelial growth factor labeling index

Multiple comparisons					
Tukey HSD					
Dependent variable	Group (I)	Group (J)	Mean difference (I-J)	SE	Significant
LI of VEGF	Dentigerous cyst	KCOT	-0.6018000	0.0850290	<0.001
		Ameloblastoma	-0.6122667	0.0850290	<0.001
	KCOT	Ameloblastoma	-0.0104667	0.0850290	0.992

HSD: Honest significant difference; VEGF: Vascular endothelial growth factor; KCOT: Keratocystic odontogenic tumor; SE: Standard error; LI: Labeling index



Graph 3: Vascular endothelial growth factor expression in ameloblastoma.



Graph 4: Quantitative analysis of vascular endothelial growth factor expression in study groups.

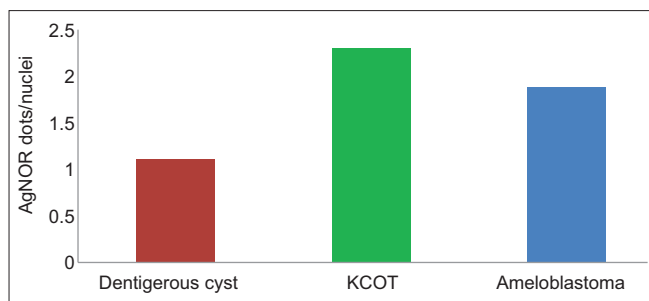
to dentigerous cysts similarly higher AgNOR count in seen in KCOTs and ameloblastoma and least in dentigerous cysts [Figure 2b].

DISCUSSION

VEGF is a potent mitogen for vascular endothelial cells and specific agent among many agents capable of initiating angiogenesis in blood vessel.^[3,10] VEGF has recently been demonstrated to play a role in proliferation of nonendothelial cells including epithelial tissues.^[6] Based on this hypothesis, we studied the expression of VEGF in cystic and neoplastic odontogenic lesions.

VEGF positivity is seen in many cases of OSCC and is used as a marker for progression and prognosis of the disease. Neoplastic cells release VEGF to promote new vessel formation by paracrine mechanism/pathway, thus provide oxygen and nutrients for tumor growth.^[11]

In our study, KCOT showed stronger VEGF expression as compared to dentigerous cyst. This is similar to the results of Mitrou *et al.*^[2] and Rubini *et al.*^[12] who also reported stronger VEGF expression by the epithelial lining of KCOT as compared to dentigerous cyst. Our results differed from those of Mitrou *et al.*^[2] in the respect that in their sample all dentigerous cysts expressed weak VEGF in the epithelium but in our study more than half the cases of dentigerous cysts were completely negative for VEGF, which is consistent with the results of Rubini *et al.*^[12] In KCOT, VEGF was expressed both in the basal as well as the suprabasal layers in all cases, but a stronger VEGF expression was seen in the suprabasal



Graph 5: Argyrophilic nucleolar organizer region counts between study groups.

cells as compared to basal cells [Tables 2 and 3]. This finding was in contrast to Mitrou *et al.* who reported similar staining intensity in all epithelial layers.^[2] In ameloblastoma, stronger VEGF expression was seen as compared to dentigerous cyst and the pattern of expression was similar to KCOT with strong expression by the stellate reticulum-like cells at the center of the follicles or strands and suprabasal layers of lining epithelium in cystic cases as compared to peripheral/basal ameloblast-like cells. Increased VEGF expression in neoplastic cells of ameloblastoma has been described by Kumamoto *et al.*,^[13] but the localization was different from our study as they showed greater VEGF expression by the peripheral cells of ameloblastoma as compared to central cells. According to Kumar *et al.*, cyclin D1 showed intense nuclear staining in both basal and stellate reticulum-like cells in both follicular and plexiform ameloblastoma.^[14] Yang *et al.* showed that there is a positive correlation between VEGF and cyclin D1 in multiple tumors.^[15] Liang *et al.* also showed positive correlation between VEGF and nonsmall cell lung carcinoma,^[16] which shows same staining pattern as seen in the present study and VEGF expression is seen in many different types of carcinomas. Hence, in the present study, the greater VEGF expression by the central cells of ameloblastoma as compared to peripheral cells may be due to several factors involved including cyclin D1. Staining in central or peripheral cells of ameloblastoma depends on various factors discussed above irrespective of type of antibody used.

Quantitative assessment of VEGF expression also showed significantly higher VEGF labeling indices for KCOT and ameloblastoma as compared to dentigerous cyst. Our results are similar to Rubini *et al.*^[12] who also showed higher percentage positivity of VEGF in the epithelial lining of KCOT as compared to dentigerous cysts. In their sample, the majority of dentigerous cysts showed less than 10%

positive cells in the epithelium while the majority of KCOTs showed more than 50% positive cells.

Leonardi *et al.* found that VEGF was expressed in the epithelial component in radicular cysts, periapical granulomas with epithelial proliferation, and rests of Malassez's while reaction of fibroblasts and inflammatory cells was heterogeneous.^[17] Graziani *et al.* found heterogeneous, weak-to-moderate expression of VEGF in the lining epithelium in radicular cysts, and strong in the connective tissue.^[18] Both studies emphasized the association of VEGF expression with inflammation and pointed to the up-regulation of cytokines that induced VEGF expression, such as interleukin (IL)-1 α , IL-6, transforming growth factor- β , insulin-like growth factor-1, in periapical lesions as a possible mechanism. Sengüven and Oygür demonstrated that IL-1 α and IL-6 are expressed in stellate reticulum-like cells in ameloblastoma, and in lining epithelial cells of KCOT. They also found a positive relationship between increased IL-1 α and IL-6 expression and tumor size. Furthermore, IL-1 α and IL-6 have been found to be produced by the epithelial cells of KCOT and ameloblastoma regardless of inflammation.^[19] Hence, it may be assumed that VEGF expression in odontogenic epithelium is not solely dependent on the presence of inflammation and, thus, some other regulating mechanisms may also exist. Stînga *et al.* reported that VEGF receptor 1 (VEGFR 1) and VEGFR2 were identified on normal structures such as canalicular epithelium of salivary glands, hair follicles, sebaceous glands, and striated muscle fibers.^[20] Cystic lesions in organs such as liver,^[6] thyroid nodules,^[21] and lung^[22] showed increased concentration of VEGF in the cystic fluid that was produced by parenchymal cells which can induce proliferation in cyst lining epithelial cells.^[6]

The lining cells also have been shown to express receptors for VEGF thus suggesting a possible autocrine mechanism where VEGF secreted by epithelial cells helps in proliferation of the same cells. The existence of similar autocrine loops with expression of both VEGF and its receptors have also been demonstrated in various tumor tissues and cell lines including those of head and neck squamous cell carcinoma.^[20,23,24] Inhibition of VEGF leading to inhibition of mitogenic activity in tumor cells/cyst lining^[6] suggests that relationship exists between VEGF and cell proliferation either directly or indirectly. Hence, it is possible that a

similar mechanism of self-stimulation may exist for odontogenic epithelial lesions also. The regulation of VEGF expression could potentially be directly influenced by the two mechanism-primary mutation or extragenetic stresses incurred by the cells. VEGF expression is induced by various cell stressors such as hypoxia, cell stretching, which may be experienced by epithelial cells of cysts.^[6] There are various factors which regulate the expression of VEGF in endothelial and nonendothelial tissues. One of these factors is β -catenin pathway, activation of which has been shown to cause increased expression of VEGF by colon cancer cells.^[25,26] Altered Wnt pathway signaling has been identified in KCOTs^[27] as well as ameloblastomas^[28] and may be one of the factors involved in expression of VEGF in these lesions.

Our results also show that mean AgNOR counts are significantly higher in KCOT and ameloblastoma as compared to dentigerous cyst [Table 2 and Graph 5] suggesting that these lesions have a higher proliferative capacity than dentigerous cyst. These findings are similar to previous studies which have shown higher AgNOR index in KCOT and ameloblastoma as compared to other cystic odontogenic lesions^[29,30] and also correlates with the greater growth potential of these lesions. The finding of a significantly higher AgNOR counts in KCOT as compared to ameloblastoma may either be incidental, given the small size of the sample, or may actually represent a difference in proliferative activity between these two lesions. A higher proliferative activity in KCOT as compared to unicystic ameloblastoma has been reported previously.^[25]

When correlating VEGF expression with AgNOR counts, it was seen that lesions showing stronger VEGF expression also showed higher AgNOR counts. Those lesions which showed VEGF expression in the basal cells had a significantly higher mean AgNOR counts than those cases showing lack of VEGF expression [Graph 4]. Similarly, mean AgNOR counts correlated positively with level of VEGF expression by suprabasal cells and the differences were statistically significant. These results show that a positive correlation exists between VEGF expression by the cystic/neoplastic epithelial cells and AgNOR counts and, hence, the proliferative capacity. A study of a larger group of these lesions comparing the solid and cystic variants separately will be required to reach an unequivocal conclusion.

CONCLUSION

We conclude that nevertheless, epithelial VEGF expression may in some way be responsible for the higher proliferative capacity of epithelial cells in KCOT and ameloblastoma, but it may also act as a mitogenic agent for epithelium of odontogenic cysts and tumors. It may also play a role in epithelial proliferation via autocrine mechanism, in addition to its established angiogenic activity via paracrine pathway and may thus be responsible for the difference in growth rate and neoplastic behavior through protein accumulation in the cystic cavity and bone resorption. Further, the similarity between KCOT and ameloblastoma, as seen in the results of the present study, reinforces the belief that KCOT exhibits characteristics of a neoplasm not only clinically but also at molecular level. The role of VEGF in the pathogenesis of KCOT and odontogenic cysts should be further evaluated using advanced techniques and more cases, as for the use of anti VEGF therapy for the management of these lesions.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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