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Impact of CD34-dependent Micro Vessel Density on Periapical Odontogenic Cysts

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Abstract. *Background/Aim: Odontogenic cysts belong to a type of lesions with endodontic origin that in some cases mimic even aggressive odontogenic tumors sharing with them similar radiographic features. Periapical cysts (PCs) belong to the inflammatory odontogenic cysts sub-category and rarely squamous cell carcinoma arises from their hyperplastic/dysplastic epithelia. This study aimed to explore the impact of cluster differentiation 34 (CD34) protein expression combined with micro vessel density (MVD) on PCs. Materials and Methods: Forty-eight (n=48) archival, formalin-fixed, and paraffin-embedded PC tissue specimens were included in the study. Immunohistochemistry (IHC) was performed in the corresponding tissue sections using an anti- CD34 antibody. CD34 expression levels and also MVD in the examined cases were measured by implementing a digital image analysis protocol. Results: CD34 over-expression (moderate to high staining intensity levels) were detected in 29/48 (60.4%) cases, whereas the rest of them (19/48-39.6%) were characterized by low levels of expression. Extended MVD was identified in 26/48 (50.1%) cases correlated with CD34 over-expression, epithelial hyperplasia (p-value=0.001), and marginally with inflammatory infiltration level in the examined lesions (p-value=0.056). Conclusion: CD34 over-expression combined with increased MVD is associated with a neoplastic-like (hyperplastic) phenotype in PCs as a result of increased neo-angiogenic activity. These histopathological characteristics rarely form an eligible substrate for squamous cell carcinoma onset in untended cases.*

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Among lesions with endodontic origin, odontogenic cysts are frequently identified and clinically categorized as developmental and inflammatory (1). According to their radiographical features they are classified as multi- or mono-unicolar cystic lesions characterized by precise and distinct borders. Interestingly, in some cases they mimic odontogenic tumors due to similar and non-discriminating radiographic features. In these cases, differential diagnosis is only available by histology (2). More specifically, inflammatory odontogenic cysts include a spectrum of sub-types such as residual, paradental, and periapical cysts (PCs), whereas developmental cysts comprise mainly gingival, eruption, glandular, keratocyst, and dentigerus, respectively. Histologically, PCs demonstrate a non-keratinized stratified squamous epithelium as a formation of one or two thin cell layers combined with fibrous connective tissue substrate infiltrated by inflammatory cells. Although rare, primary intraosseous squamous cell carcinoma arises from their hyperplastic/dysplastic epithelia in untended, misdiagnosed cases (3, 4).

Hyperplasia in a fragment of PCs is correlated with increased inflammation and angiogenesis. In order to evaluate different neo-angiogenesis levels inside malignant tissues derived from oral and odontogenic epithelia, many studies have focused on cluster differentiation 34 (CD34) protein expression combined with micro vessel density (MVD) in them (5-9). CD34 (gene band: 1q32.2) is a ~120-kDa cell surface-transmembrane phosphoglycoprotein acting as an adhesion molecule between cells (10). Naive stem hematopoietic, vascular-associated progenitor and also mature murine mast

cells express the marker in high levels (11-13). Furthermore, CD34 enhances T-cell motivation leading to lymph node infiltration (14). Concerning MVD measurement in PCs, CD34 is a sensitive and specific marker based on immunohistochemistry (IHC) protocols implementation in a variety of tissue substrates including cysts and neoplastic lesions such as ameloblastomas and oral carcinomas (15, 16). In the current experimental, research study, we explored the impact of CD34 protein expression combined with MVD on a PCs series using modern techniques.

Materials and Methods

Study group. A series of forty-eight (n=48) archival, formalin-fixed, and paraffin-embedded PC tissue specimens were analyzed. Among patients, 26 were males, whereas the rest of them (n=22) females. The National and Kapodistrian University Medical Ethics Committee consented to the use of these tissues in the 1ST Department of Pathology, Medical School, National and Kapodistrian University, Athens, Greece for research purposes, according to World Medical Association Declaration of Helsinki. The tissue samples were fixed in neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO) for oral and especially odontogenic lesions (17).

Antibodies and IHC assay. Ready-to-use mouse monoclonal anti-CD34 (clone QBEnd10, Dako, Glostrup, Denmark) antibody was selected at a dilution of 1:200. The IHC assay was implemented on 4 μm serial tissue sections obtained from the corresponding tissue blocks. Firstly, the slides were de-paraffinized followed by rehydration. According to the manufacturer's guidelines, the hyperoxide/streptavidin/biotin protocol (Dako) combined with EnVision FLEX TBS (Tris-buffered saline: 0.05 MTris/HCl, 0.15 MNaCl, pH=7.6) wash Buffer (20×) was applied. Diaminobenzidine-tetrahydrochloride (DAB–Dako) containing 0.1% hydrogen peroxide was used as a chromogen. After its incubation, the tissue sections were counterstained using Hematoxylin, for 5 min, dehydrated, and finally cover-slipped. The primary antibody was omitted in the case of control slides. An automated staining system (Biogenex, Fremont, CA, USA) was used for the current IHC procedure. Endothelial cell continuous, membranous staining was considered suitable for the marker, according to manufacturers' data sheets (Figure 1A). Normal endothelia expressing the protein were used as the control group.

Digital image analysis (DIA) assay. CD34 protein expression levels and MVD levels were estimated quantitatively by measuring the corresponding CD34 staining intensity levels (densitometry calculation) in the stained endothelial cells and their number, respectively. A DIA assay was implemented based on a semi-automated system (hardware: Microscope CX-31, Olympus, Melville, NY, USA; Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp., Tokyo, Japan). According to the digitized algorithm, CD34-related stained areas were detected (5 optical fields at ×400 magnification) and a digital database including the corresponding snapshots was constructed. A specific macro (membranous expression pattern) was assessed as a matrix for the measurements. Based on an algorithm, an extensive spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) color spectrum was eligible for calculating different protein expression levels (Figure 1B). Staining intensity values decreasing to 0 correspond to a progressive protein over-expression. In contrast, increased values to 255 lead a progressive loss of its staining intensity. MVD levels were measured as a number of CD34-stained endothelial ring-like structures (vessels) per high power optical field. Total results for CD34/MVD and DIA values are demonstrated in Table I.

Statistical analysis. In order to analyze statistically the extracted results, we applied the statistics software package Statisticav. 6.0 (StatSoft Power Solutions Dell, TIBCO Soft, Palo Alto, CA, USA). Quantitative variables were presented as mean±standard deviation, whereas qualitative variables were presented in frequency tables. To evaluate the relationship between qualitative and quantitative variables, because of the small number of subjects in each group, the nonparametric Mann–Whitney and Kruskal–Wallis tests were applied. To evaluate the relationship between independent qualitative variables, where appropriate, the chi-square test for linear trend and Fisher exact test were applied. Statistical significance (p) was evaluated in pairs and differences <0.05 were considered statistically significant. Total IHC results and differences (p-values) are described in Table I.

Results

All examined PC cases expressed the marker in different levels. CD34 over-expression (moderate to high staining intensity levels) was detected in 29/48 (60.4%) cases, whereas the rest of them (19/48-39.6%) were characterized by low levels of expression. Increased MVD was identified in 26/48 (50.1%) cases -as an absolute number of endothelial ring-like structures -and correlated with CD34 over-expression, epithelial hyperplasia (p-value=0.001), and marginally with inflammatory infiltration level in the examined lesions (p-value=0.001), whereas no correlation was established with sex (p-value=0.534).

Discussion

Approaching the non- or neoplastic dental lesions, periodontitis is a frequent and progressively severe inflammation mediated by bacteria accumulation combined or not with other factors such as low-level mouth hygiene conditions and tobacco chronic consumption (18). More specifically, its onset includes gingivitis presenting clinical signs such as swollen gums, bleeding, plaque, and teeth pain. Besides periodontitis, peri-implantitis is characterized by similar clinical signs Interestingly, there is a genetic predisposition in specific populations (19). Furthermore, chronic colon inflammation (colitis) leads indirectly to periodontitis by altering specific metabolic pathways (20). Additionally, *Helicobacter pylori* (*H. pylori*) stomach infection was suggested to be present in chronic periodontitis patients,

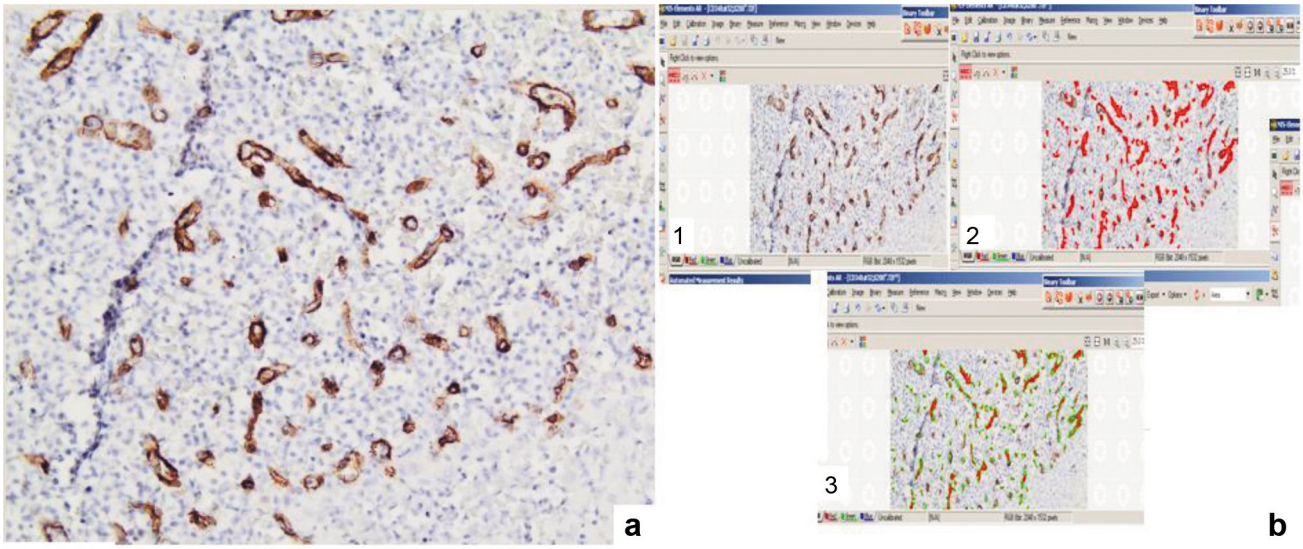

Figure 1. *CD34-dependent micro vessel density (MVD) in periapical cyst tissues A) A PC case presenting CD34 over-expression and increased MVD. Note the brown continuous strong staining pattern (>15) in an endothelial ring like vessels B) Implementation of a DIA assay for estimating MVD rates with CD34 over-expression. The process is divided in three stages (1-3): first digitized snapshot image phase; second, marking phase using a digital red paint; and third, a measurement phase with red/green vessels' encircling and calculating MVDs in the examined cases (original magnification 400×, MVD, DAB brown chromogen for immunohistochemistry protocol).*

Table I. *Total CD34 immunohistochemistry and micro vessel density (MVD) results and statistics.*

Clinicopathological parameters		CD34 expression		*p*-Value
PCs (n=48)		OE	L	
	n (%)	29/48 (60.4%)	19/48 (39.6%)	
Sex				0.534
Male	26/48 (54%)	17/48 (35%)	9/48 (19%)	
Female	22/48 (46%)	12/48 (25%)	10/48 (21%)	
Epithelial hyperplasia				**0.001***
Hyperplastic	23/48 (48%)	21/48 (44%)	2/48 (4%)	
Non-hyperplastic	25/48 (52%)	8/48 (17%)	17/48 (35%)	
Inflammation				0.056*
Inflammatory infiltrated	21/48 (44%)	14/48 (29%)	7/48 (15%)	
Non-inflammatory infiltrated	27/48 (56%)	15/48 (31%)	12/48 (25%)	
MVD				**0.001**
H	26/48 (54%)	25/48 (52%)	1/48 (1%)	
L	22/48 (46%)	4/48 (9%)	18/48 (38%)	

*MVD *vs*. epithelial hyperplasia, MVD *vs*. inflammation. PCs: Periapical cysts; CD34 OE: Over-expression, staining intensity values ≤117 (spectrum between 109 and 117), L: Low, staining intensity values >139 (spectrum between 129 and 142); MVD H: High rates >15 micro vessels per optical field, L: Low rates <15 micro vessels per optical field. *p*-Values in bold indicate statistically significant differences.

but their exact relation is under investigation (21). In conjunction, apical periodontitis is the result of chronic endodontic inflammation mediated by bacteria of the *Porphyromonas* spp. including predominantly *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* (22, 23).

Odontogenic cysts - combined or not with periodontitis - represent frequent lesions in adults. Referring to PCs, they are characterized histologically by non-keratinized stratified squamous epithelium including thin (one or two) layers combined with excessive fibrous connective tissue and

inflammatory infiltration. Interestingly, there is a variety of non-malignant non-endodontic periapical lesions (NMNPLs) that mimic pure PCs (24). Additionally, pathological entities that share similar histological characteristics with PCs include squamous odontogenic tumors and non-neoplastic lesions with epithelial hyperplasia inside a radicular cyst (25).

In this study, we explored the role of CD34 protein expression combined with MVD in a series of PC tissues using IHC and DIA methods. We observed that CD34-dependent MVD was increased significantly in cases with epithelial hyperplasia and marginally with inflammatory infiltration. It seems that PCs incorporating these specific histological features demonstrate a more aggressive biological behavior (increased angiogenesis). Another study co-analyzed receptor for advanced glycation end products (RAGE), S100, and CD34 molecules in a series of periapical granuloma tissues (26). They reported elevated CD34 protein expression leading to endothelial hyperplasia (increased angiogenesis) in these inflammatory cystic lesions. Similarly, interleukin (IL)-17 – a molecule that acts as a cytokine- has been found to be over-expressed in apical periodontitis lesions (periradicular cysts and granulomas) in conjunction with CD34 (27). Both of them promote inflammation and angiogenesis in them, respectively. CD34 over-expression is also observed in regenerative endodontic processes providing healing of periradicular lesions (28). Furthermore, quantitative digital analysis of MVD rates in PCs and other oral-dental lesions is superior compared to conventional eye-based evaluation. In the current study, we estimated MVD levels in a fast and accurate way using a digitized algorithm based on CD34 IHC expression levels. Our previous studies on oral malignancies are concordant with similar experimental studies (29-35). All of them suggest and enhance this practice because it provides a systematic screening and mapping of immunostained slides.

In conclusion, CD34 over-expression combined with increased MVD is correlated with a neoplastic-like (hyperplastic) phenotype in PCs as a result of increased neo-angiogenic and inflammatory activity. A subset of PCs characterized by elevated MVD rates demonstrate significant epithelial hyperplasia and increased inflammatory infiltration, mimicking neoplastic lesions. These histopathological characteristics rarely form an eligible substrate for squamous cell carcinoma onset in untended cases.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

VM, ET: design of the study, ET, VM: manuscript writing, IT, DP, ACL, NK: academic advisors: SM: collection and management of references' data. All Authors read and approved the final manuscript.

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