CORRELATION BETWEEN AMELOGENIN AND OSTEOPONTIN EXPRESSION IN AMELOBLASTOMAS AND ODONTOGENIC CYSTS: AN IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Background and Aim: Odontogenic tumors are heterogeneous group of lesions originating from odontogenic apparatus and their remnants. Although their etiopathogenesis remains unclear, some advances have been accomplished in understanding the role of their enamel-related proteins. Amelogenin is the major structural enamel matrix protein involved in the development of odontogenic epithelium. Osteopontin is a multifunctional biomarker that affects cell survival, migration, calcification inhibition, and immune cell function modulation. The study aimed to assess the immunoexpression of both biomarkers in different odontogenic lesions.

Material and Methods: Forty-eight paraffin-embedded blocks of odontogenic tumors and cysts were involved including multicystic ameloblastoma (n=10), unicystic ameloblastoma (n=9), dentigerous cyst (n=11), odontogenic keratocysts (n=7), and radicular cyst (n=8). Quantitative immunohistochemical analysis for amelogenin and osteopontin were recorded with index of positivity. The results were statistically analyzed using Kruskal-Wallis test and Spearman's coefficient rank correlation analysis.

Results: Positive immunoreaction for amelogenin was demonstrated in both control and dentigerous cyst groups while unnoticeable expression was detected in other studied groups. Regarding osteopontin, positivity was detected in ameloblastomas and some examined cysts. Highly statistically significant differences between both biomarkers were revealed in all tested groups (p < 0.001). Spearman's rank analysis reported negative correlation between both biomarkers with highly significant values (p < 0.0001).

Conclusion: Amelogenin can be a promising predictor for the biological behavior of odontogenic lesions and pro-invasive factor osteopontin can predict neoplastic potential and high probability of recurrence.

KEYWORDS: Amelogenin, Osteopontin, Index of Positivity, Ameloblastoma, Odontogenic Cyst.

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INTRODUCTION

Odontogenic lesions are a category of heterogeneous lesions that develop from the tooth-forming structure residues, which include epithelial, ectomesenchymal and mesenchymal remnants [1]. These lesions can have a wide range of proliferative behaviours, such as hamartomas or neoplasms and they still have an attractive inquiry era of research and investigation [2].

One of the most frequent odontogenic tumors is the benign ameloblastoma (AB) which demonstrate either central or peripheral occurrence with two subtypes of central ABs which are multicystic/solid and unicystic. It is considered as a locally aggressive odontogenic tumor with a greater chance of recurrence following surgery, which makes up around 10 % of all neoplasms in the jaws and it is hypothesized to originate from a variety of odontogenic epithelial sources [3-5].

The most prevalent type of cystic lesions affecting the maxillofacial region is odontogenic cysts that generally divided into two groups an inflammatory group, which includes radicular cysts (RCs) and a developmental group, which includes dentigerous cysts (DCs) and odontogenic keratocysts (OKCs) [6, 7].

According to the latest edition of the WHO in 2017, RCs are considered the most common odontogenic cysts of the jaws in literature followed by DCs and then OKCs. This high incidence of RCs may be attributed to the precarious oral states and the deficient public awareness to hinder oral infectious diseases [8].

Basically, DCs are the most frequent developmental odontogenic cysts which mostly inhibit the eruption of teeth, build-up of fluid between the reduced enamel epithelium and the tooth crown is assumed to be the reason. It is usualluy linked with impacted or un-erupted teeth [3].

OKC is a form of odontogenic cyst that has a parakeratotic epithelial lining. The WHO termed it keratocystic odontogenic tumor due to debate in its severe clinical behaviour and neoplastic nature, however the term was eventually changed back to OKC in 2017. After surgical treatment, OKCs are well-known for their high recurrence rate and bone destructive nature. However, the molecular mechanisms that underpin local aggressive behaviour and OKCs' significant osteolytic potential are still unclear [9].

Amelogenin (AMEL) isolated by Termine et al. [10], is extremely important for the organization and mineralization of developing enamel. As a result, AMEL expression is thought to be a predictor of epithelial cell differentiation in odontogenic lesions. Furthermore, AMEL proteins are required for the production of enamel in teeth and may play a role in managing and regulating the finely woven hydroxyapatite microstructure [11].

Furthermore, identifying AMEL expression might help researchers for better understanding the pathophysiology of lesions and predict the histological behaviour and clinical characteristics of odontogenic tumors or cysts [1]. The AMEL proteins are largely encoded by the AMEL-X gene on the X chromosome. In men, the AMEL-Y gene on the Y chromosome is thought to be 10 times more active than AMEL-X in generating AMEL proteins [12].

Interestingly, Osteopontin (OPN) is a compound word that combines the words "osteo" and "pontin" to describe its role as a link between bone cells and the bone extracellular environment. In 1979, it was categorized as a secretory phosphoprotein 1 (SPP1) for the first time [13]. OPN is a 34 kDa acidic glycoprotein that undergoes post-translational changes following cellular secretion, resulting in molecular sizes ranging from 44 kDa to 75 kDa in mammalian cells. OPN is categorized as a member of the SIBLING (Small Integrin-binding Ligand N-linked Glycoprotein) family [14].

OPN has been found to have multiple functions, including cell migration, cell survival, calcification

inhibition, immune cell function regulation, and tumor cell phenotype control [15]. In addition, OPN is abundantly expressed in osteoblasts and osteoclasts, where it plays a significant role in biomineralization. It also engages in numerous physiological activities of homeostasis and pathology, such as chronic inflammation and tumor biology [16].

Accordingly, this study was carried out to assess and correlate the immunoexpression of AMEL and OPN biomarkers in ABs and various odontogenic cysts including RCs, DCs and OKCs.

MATERIAL AND METHODS

Study Design and Sample Size

This retrospective case control study was carried out in accordance with the principles of the Helsinki Declaration and following the STROBE Checklist. The archival blocks of Oral and Maxillofacial Pathology Department, Faculty of Dentistry, October 6 University were reviewed from January 2010 to October 2020 for cases of odontogenic tumors and cysts to be assessed for eligibility for implication in the current work. The clinical records of patients regarding age, gender, and location of the lesions were obtained from the electronic medical record (EMR) software system.

Similar studies [4, 12] in terms of research questions and methods were used for calculation of sample size where the ones with the highest level of evidence selected as an evidence-based measure of effect to perform power analysis for estimating the minimum number of cases to be used in the study (significance level type1 error as 0.05 and power type 2 error as 0.8) using statistical G* Power application software version 3.1.9.7. It was determined that estimated sample size consisted of approximately 35 samples is adequate to detect significant differences.

A total of 48 paraffin-embedded blocks were selected to conduct the current study. The included cases were 26 odontogenic cysts [8 RC, 11 DCs and 7 OKCs], 19 odontogenic tumors [10 multicystic ABs subdivided histologically into 2 acanthomatous ABs and 8 follicular ABs in addition to 9 unicystic ABs classified into 3 cases of mural type and 6 cases of luminal type]. Three samples of control tissues for odontogenic epithelium from dental germs were acquired after surgical removal of various jaw lesions due to their proximity.

The inclusion criteria were cases that fulfilled the histological features described in the most recent classification of odontogenic cysts and tumors of the WHO [17]. On the contrary, inflammation at the interface of the fibrous capsule and the lining epithelium of developmental cysts and OKCs linked to nevoid basal cell carcinoma syndrome were excluded. Demographic clinicopathological data of examined lesions were summarized in Table 1.

TABLE (1) Summation of demographic data of the involved studied cases.

Lesion	No. of Samples	Age range/Sex	Site
DC	11	18-26 years 7 male/4 female	Mandible=11
OKC	7	22-57 years 5 male/2 female	Mandible=6 / Maxilla=1
RC	8	16-45 years 4 male/4 female	Mandible=3/ Maxilla=5
Multicystic AB	10	24-55 years 7 male/3 female	Mandible=10
Follicular	8		
Acanthomatous	2		
Unicystic AB	9	22-50 years 6 male/ 3 female	Mandible=9
Luminal	6		
Mural	3		

Three sections of 4 μm were cut from each block, one stained with routine Hematoxylin and Eosin (H&E) for confirmation of the previous diagnosis. The other two sections were used for immunohistochemical assessment.

Immunohistochemical Staining

The standard method (streptavidin-biotin peroxidase) was used for staining procedures. Four-micrometer tissue sections were cut from the paraffin blocks and loaded on positively charged glass slides (Optiplus, Biogenex, USA) for immunostaining with antibodies for AMEL (Thermo Scientific, Lab vision, USA) and OPN (BIOCYC GmbH & Co. KG, Guartett, Germany). The tissue sections were deparaffinized in xylene before being hydrated in descending alcohols then rinsed in running water for 10 min At room temperature, tissue sections were immersed in 3 % hydrogen peroxide for 30 min. to suppress endogenous peroxidase activity.

The mounted slides were boiled in citrate buffer (10 mM - pH 6.0) for 20 min for antigen retrieval followed by cooling in the room. The sections were then incubated overnight at 4°C with the appropriate dilutions of primary antibodies. After washing the slides in phosphate buffer solution (PBS), the biotin-labeled link antibody was applied, followed by streptavidin-conjugated horseradish peroxidase.

Tissue sections were incubated with primary antibodies for 20 min at 37°C, followed by incubation with the biotinylated secondary antibody for another 20 min at 37°C. Diaminobenzidine chromogen was used to visualize the antigen-antibody reaction. All samples were counterstained with Mayer's hematoxylin before being coated with coverslips using an aqueous mounting technique. Negative controls were performed using the same procedure, but without the main antibody and instead treated with PBS.

Immunohistochemical Evaluation

Localization and detection of the immunostaining of the two biomarkers expression in the epithelial components of the lesions were undertaken using an ordinary light microscope (Leica, Germany), where cells with cytoplasmic staining were considered positive. The quantitative immunohistochemical analysis for AMEL and OPN was recorded as an index of positivity (IP) for each used biomarker. Image Analysis for IP was performed using the Leica Q550 IW Imaging Workstation and reported by the percentage of cells.

The proportion of positively stained cells for each section was calculated by the number of marked cells per total number of 500 cells identified at magnification (x 400) followed by multiplying the result by 100 (AMEL and OPN positive cells /500) x 100). The percentage was rated on a scale from 0 to 3 as follows: 0: No identified or unnoticeable staining of the epithelium components; 1: < 10 % staining; 2: 10 – 50 % staining; 3: < 50 % staining [18] .

Statistical Analysis

All the collected data were analyzed using SPSS (Statistical Package for Social Sciences) 26.0 software (IBM, Chicago). The distribution of data was evaluated through descriptive statistics which have assessed the abnormality of data. The Kruskal-Wallis nonparametric test was used to compare different levels of expression among groups, followed by post-hoc pairwise comparison using the Bonferroni method. The correlation between the OSN and AMEL expression was assessed using Spearman's rank correlation analysis. A two-tailed P value less than 0.05 was considered statistically significant.

RESULTS

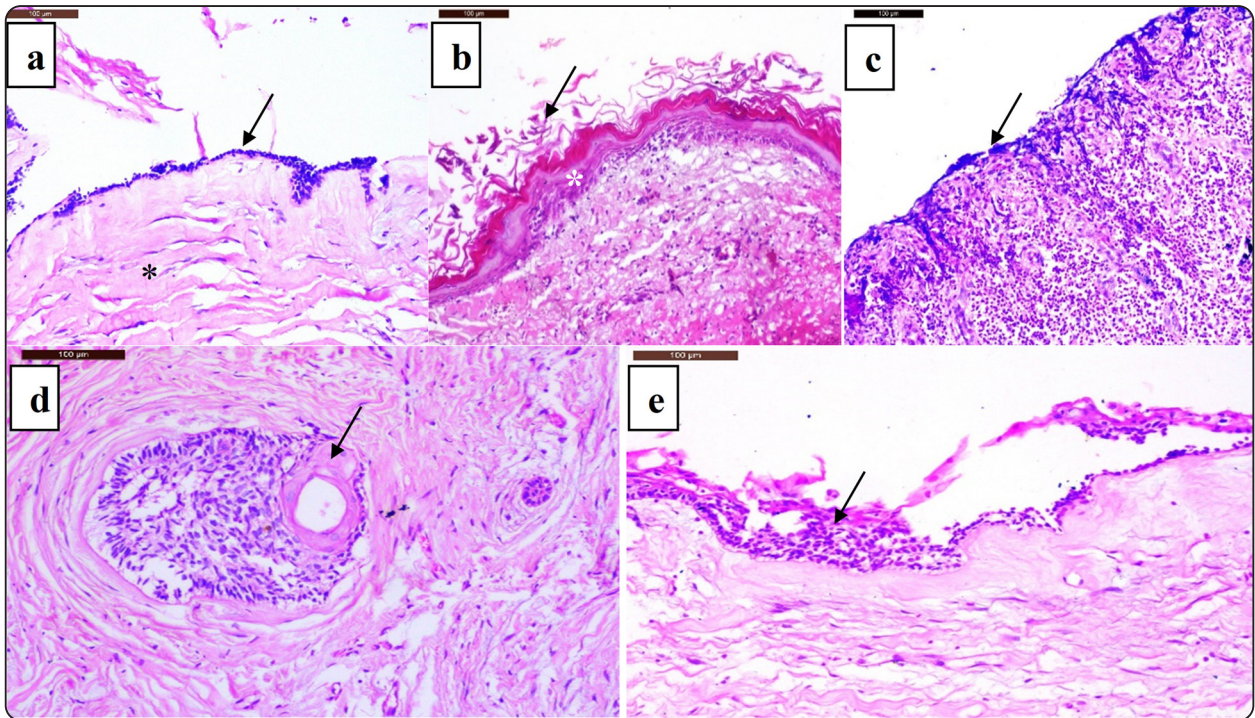Histopathological examination of H & E stained sections confirmed the previous diagnosis (Figure 1 a - e).

Fig. (1) Photomicrographs of DC (a) showing 2-4 layers of epithelium lacking superficial keratinization (arrow) and fibrous connective tissue capsule (*). OKC (b) with a uniform epithelial lining devoid of rete ridges and a palisaded hyperchromatic basal cell layer comprising cuboidal to columnar cells (*) and corrugated parakeratotic epithelial cells cover the luminal surface (arrow). RC (c) with thin flattened stratified squamous epithelial lining (arrow), less vascularized thick mature fibrous wall, and heavy inflammatory infiltrate. Follicular AB (d) demonstrating acanthomatous variant in which island of odontogenic epithelium in fibrous stroma with squamous metaplasia of stellate reticulum cells and variable keratinization (arrow). Unicystic AB (e) showing ameloblastomatous epithelium lining part of cystic cavity with luminal proliferation (arrow), (H & E x 400).

Amelogenin (AMEL) Immunoexpression

Expression of AMEL was represented as cytoplasmic brown staining in the epithelial components with variable percentages of IP. Immunoreactivity was identified in control samples, the tooth germ ameloblasts showing highest positivity and the odontoblasts showing varied positivity. All cases of DCs demonstrated positive reaction in the lining epithelium and scarce stromal cells in the surrounding capsule. In addition, only one case of OKCs (1/7) and RCs (1/8) appeared with focal positive reaction limited to the lining epithelium. However, negative reaction or unnoticeable staining was observed in all cases of the ABs with its different histological types, (Figure 2 a-f, Table 2).

Osteopontin (OPN) Immunoexpression

Control sections displayed negative immunostaining of OPN in the epithelial cells. Seven cases of DCs (7/11), two cases of OKCs (2/7) and four cases of RCs (4/8) demonstrated no identified reaction with OPN in lining epithelial lining while in remaining cases epithelial cells, stromal cells, and endothelial cells all showed cytoplasmic immunoreactivity. Meanwhile, higher percentages of IP in OPN staining were noted in all studied cases of ABs, multicystic ABs showed an intense cytoplasmic reaction in neoplastic ameloblast-like cells and stellate reticulum like cells, in addition, stromal positivity was also noted. In luminal types of unicystic ABs, cytoplasmic response was observed in tumor cells and inflammatory cells in
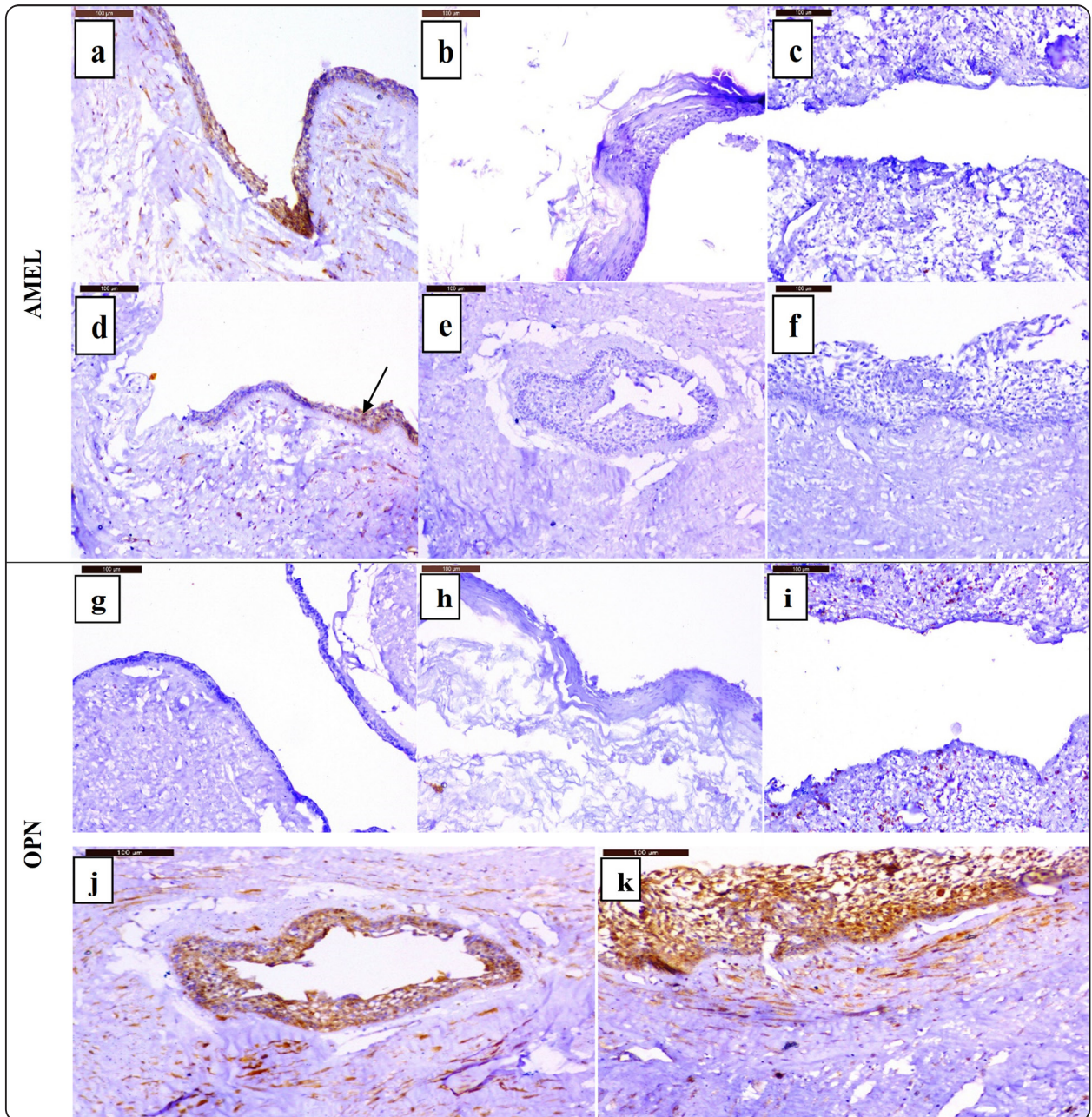
Fig. (2) Photomicrograph of AMEL expression showing positive immunoreactivity in the lining epithelium of DCs (a), while the negative reaction was observed in OKCs (b), RCs (c), follicular AB (d) and unicystic AB (e). RC demonstrated localized positive reaction (arrow) in some areas of lining epithelium (f), (x 400). Photomicrograph of OPN expression showing negative immunostaining in the lining epithelium of DCs (g), negative reaction was revealed in OKCs (h) and in RCs (i), while positive reaction was observed in follicular AB (j) and unicystic AB (k), (x 400).

the stroma. Meanwhile, mural type of unicystic ABs demonstrated tumoral and peritumoral positive immunoreaction. Furthermore, subepithelial stromal inflammatory cells showed localized OPN immunopositivity of RCs (Figure 2 g - k, Table 2).

Statistical Results

Differences in AMEL and OPN immunoreactivity as represented by the score of IP was found to be significantly higher in all tested groups (p < 0.001), (Table 2). The values of the Chi- Square test confirm the significance of observations obtained in the Kruskal–Wallis test, (Chi- square = 40.205 for AMEL and 44.207 for OPN, df = 5, p < 0.001). On the contrary, post-hoc pairwise comparison using the Bonferroni method demonstrated that differences were significantly higher only in unicystic and multicystic ABs, (Table 3). Spearman's coefficient rank analysis between OPN and AMEL reported moderate negative correlation with highly significant two- tailed p-values (r = $-$ 0.58 1, p < 0.0001), (Figure 3).

TABLE (2) The percentage of positive cases and immunohistochemical staining score of AMEL and OPN in terms of index of positivity (IP) in the study groups and mean rank with Kruskal-Wallis test.

Biomarkers	Lesions (n.)	Positive Cases (n., %)	Scoring (n., %)				Mean Rank	Kruskal-Wallis H	p-value
			0 (Score=0)	**< 10 %** (Score=1)	**10 – 50 %** (Score=2)	**> 50 %** (Score=3)			
AMEL	Control n=3	3 (100 %)	0 (0 %)	0 (0 %)	1 (33.4 %)	2 (66.6 %)	41.67		
	DC n=11	11 (100 %)	0 (0 %)	0 (0 %)	4 (36.6 %)	7 (63.4 %)	41.45		
	OKC n=7	1 (14.3 %)	6 (85.7 %)	1 (14.3 %)	0 (0 %)	0 (0 %)	18.93		
	RC n=8	1 (12.5 %)	7 (87.5 %)	1 (12.5 %)	0 (0 %)	0 (0 %)	18.63	42.178	0.0001
	Multicystic AB n=10	0 (0 %)	10 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)	16.5		
	Uniystic AB n=9	0 (0 %)	9 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)	16.5		
OPN	Control n=3	0 (0 %)	3 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)	8.5		
	DC n=11	4 (36.6 %)	7 (63.4 %)	4 (36.6 %)	0 (0 %)	0 (0 %)	12.68		
	OKC n=7	5 (71.5 %)	2 (28.5 %)	2 (28.5 %)	3 (42.8 %)	0 (0 %)	19.71		
	RC n=8	4 (50 %)	4 (50 %)	1 (12.5 %)	3 (37.5 %)	0 (0 %)	18.88	38.627	0.0001
	Multicystic AB n=10	10 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)	10 (100 %)	39.5		
	Uniystic AB n=9	9 (100 %)	0 (0 %)	0 (0 %)	1 (11.1 %)	8 (88.9 %)	38.11		

TABLE (3) Bonferroni post-hoc test for pairwise comparing between and within studied groups.

Group 1	Group 2	Test Statistic (Group 1-2)	Std. Error	Std. Test Statistic	P	Adj. Sig.[a]
Control	DC	-4.182	8.672	-0.482	0.630	1.000
	RC	-8.375	9.014	-0.929	0.353	1.000
	OKC	-11.214	9.188	-1.221	0.222	1.000
	Unicystic AB	-29.611	8.876	-3.336	0.001	0.013
	Multicystic AB	-31.000	8.764	-3.537	0.000	0.006
DC	RC	4.193	6.187	0.678	0.498	1.000
	OKC	-7.032	6.437	-1.092	0.275	1.000
	Unicystic AB	-25.429	5.984	-4.249	0.000	0.000
	Multicystic AB	-26.818	5.817	-4.610	0.000	0.000
RC	OKC	-2.839	6.891	-0.412	0.680	1.000
	Unicystic AB	-21.236	6.470	-3.282	0.001	0.015
	Multicystic AB	-22.625	6.315	-3.582	0.000	0.005
OKC	Unicystic AB	-18.397	6.710	-2.742	0.006	0.092
	Multicystic AB	-19.786	6.561	-3.016	0.003	0.038
Unicystic AB	Multicystic AB	-1.389	6.117	-0.227	0.820	1.000

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.
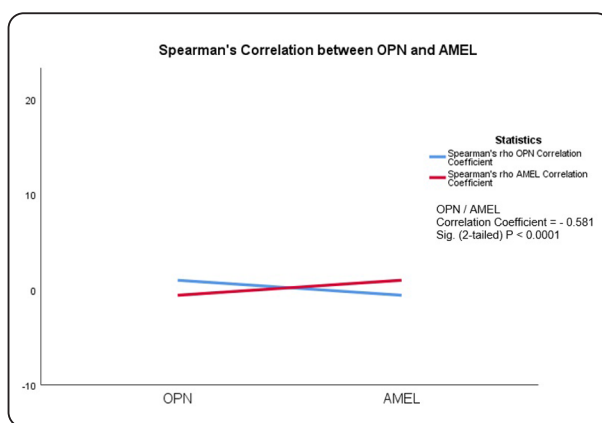

Fig. (3) Graphical representation of Spearman's correlation coefficient (Spearman's rho) between OPN and AMEL.

DISCUSSION

Odontogenic tumors and cysts are considered as a wide variety of lesions that emerge from the odontogenic apparatus and its remnants with varying degrees of aggressiveness [6]. The quantity of epithelium in most odontogenic lesions varies obviously, and particular studies revealed that aberrations of enamel-related proteins play a role in the oncogenesis of the odontogenic epithelium [19].

Ameloblasts generate AMELs and non-AMELs, which are important protein components in the enamel matrix. AMEL was discovered in the enamel organ's cellular cytoplasm. Localization of AMEL immunoexpression may help in understanding the pathogenesis of odontogenic lesions, as well as the prediction of their histological behaviour and clinical characters [20].

Observations of the current study revealed no correlation between AMEL and OPN expression and demographic features of the analyzed cases such as age, sex, and site which were similar with the findings of many reviewed authors.

Regarding the current AMEL results in all cases of ABs, our findings revealed a negative reaction or unnoticeable staining in all histological types, which was consistent with the findings of Saku et al. [21] and Crivelini et al. [22], who found that

AMEL was expressed only in odontogenic lesions with mineralized tissues, such as adenomatoid odontogenic tumors (AOT) and calcifying epithelial odontogenic tumors (CEOT) and stated that positive staining was limited to the mineralized foci.

These findings are possibly due to the undifferentiated nature of AB which is hypothetically derived from ameloblasts of a pre-secretory lineage that are immature cells to express a detectable amount of enamel proteins or show mineralization activity. This could explain the lack of expression of these proteins as well as the absence of calcified structures in these odontogenic tumors.

In the same context, the results of Anigol et al. [12] of AB have partially supported our findings as half of their cases showed a negative reaction. Conversely, their results of reduced enamel epithelium and dental lamina were with ours of dental germs. On the contrary, Zakaraia et al. [1] and Urzua et al. [23] reported positive AMEL immunoexpression in ABs.

Moreover, considering immunoexpression results of Anigol et al. [12] in DCs and RCs, just one of their cases showed a positive reaction, and this was nearly consistence with our results. Furthermore, the findings of Zakaraia et al. [1] were consistent with the current results of DCs, OKCs, and RCs, as they declared OKC positivity and their rationale was elucidated by the fact that AMEL expression is likely indicative of epithelial cell differentiation in odontogenic lesions. Moreover, they stated that AMEL stimulates the functioning of mature ameloblasts with low proliferation capacity being present in low aggressive odontogenic lesions.

AMEL was noticeably regarded to be a biomarker for odontogenic epithelium and lesions, especially when routine H&E stains failed or found it difficult to identify these lesions. It was recently proven that the degree of AMEL expression in odontogenic lesions is varied, which might explain how the cells differentiate at different stages of their journey through the lesions [24].

Furthermore, a remark of nonspecific staining was linked to AMEL's multifunctional activity in various tissues throughout embryologic craniofacial development in the study conducted by Green et al. [25]. Surprisingly, the AMEL molecule has the capacity to anticipate how odontogenic lesions will behave [9].

Elevated expression of OPN in AB tumor cells and peritumor stromal cells might explain multicystic AB and mural unicystic AB's locally invasive nature and strong osteolytic potential. OPN can promote tumor cell motility, invasion, and dissemination, as well as activate osteoclasts and protect cells from immune-mediated cytotoxicity. As a result, there was a difference between the same lesions in the same investigations, which may be explained by the sensitivity of the antibody employed in each study as well as ethnic differences in tumors [26].

Regarding immunohistochemical findings of OPN in the current study, high percentages of IP were reported in all studied cases of ABs. The results of Wang and Liu [27] and Masloub et al. [4] agreed with our observations, they showed positive reactions in ABs cases especially in follicular type with OPN immunoreactivity was seen in the cytoplasm of ameloblast-like cells, with just a few cytoplasmic localizations in stellate reticulum-like cells.

Peritumoral response was also observed in the stroma around the tumor. Wang and Liu [27] corroborated and explained this data, claiming that OPN generated by tumor cells can improve tumor cell adhesion and bone migration, leading to tumor dissemination and invasion. The tumor cells and inflammatory cells in the stroma of luminal unicystic ABs displayed a cytoplasmic reaction, but mural unicystic ABs showed tumoral and peritumoral positive immunoreaction.

In ABs, OPN protein is most likely generated and produced by stellate reticulum-like cells, which are subsequently scooped up and dumped into the peritumor stromal tissue by ameloblast-like cells (ameloblast-like cells transcytosis) [27, 28]. The locally invasive character of multicystic AB and mural unicystic AB, as well as their strong osteolytic potential, might be explained by increased expression of OPN in AB tumor cells and peritumor stromal cells. OPN has been shown to aid tumor cell migration, invasion, and dissemination, as well as to activate osteoclasts and shield cells against immune-mediated cytotoxicity.

In the present work, we observed positive OPN reaction in some cases of DCs (4/11), OKCs (5/7) and RCs (4/8) which came in the same context with the results of Wang and Liu [27] and Wang and Liu [28] as they reported almost negative reactions of DCs and RCs with considering focal weak positivity only in one case of RCs and strong positive reaction in majority of OKCs cases. This pattern of OPN expression in DC epithelial lining could be used as an early predictor of DC neoplastic transition into unicystic AB. Induced OPN expression has been observed in epidermal cells during remodeling processes as the tumor advances, which supports this theory [29]. Chang et al. [30] backed up this claim that OPN expression is linked to tumorigenesis in both benign and malignant tumors.

In full agreement with our findings, Kechik and Siar [31] results showed under-expression and indistinctive distribution patterns of OPN among all studied samples of three types of cysts RCs, DCs and OKCs. Similarly, our observations of OPN concurred with the findings of existing studies of Salehinejad et al. [32] and Woźniak et al. [33] who reported absent or weak expression of this antibody in RCs and DCs. This was clarified by that OPN could be well expressed in non-malignant tissues because of its multiple physiological activities.

CONCLUSION

Based upon the findings of this research, it may be inferred that AMEL varies at different stages of a lesion's development after a functional alteration and that it is controlled by environmental and genetics factors. Therefore, the AMEL molecule holds abundant promising predictors for better understanding the pathophysiology, biological behavior of aggressiveness and clinical characteristics of odontogenic lesions. On the other hand, OPN also plays a role as a pro- invasive factor, with its expression being able to predict neoplastic potentiality and serve as a tool for identifying lesions with locally invasive behaviour and a high probability of recurrence.

Limitation of the Study

A certain incarceration of our study was the limited availability of archival samples of different types of odontogenic lesions. To support the detailed observation of the biological activity of the examined molecules, more research with a great number and diversity of samples is necessary.

Ethical Statement

The current study was authorized by Research Ethics Committee at Faculty of Dentistry, October 6 University (No. RECO6U/2-2021) and carried out in accordance with the principles of the Helsinki Declaration. Formal written informed consent was not required for this study with a waiver by the Research Ethics Committee.

Declaration of Competing Interest

All authors declared no potential conflicts of interest with respect to the research, authorship and publication of the article.

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Authors' Contributions

All authors had substantial contributions in the conception and design of the current study, where NFH performed analysis and interpretation of data, YAE carried out collection and assembly of data, and AFF provided administrative support and provision of study materials. Drafting and revising the manuscript critically for important intellectual content and approval of manuscript submission in its current form were committed by all authors.

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