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COMPARATIVE STUDY OF BEHAVIOR BETWEEN CONVENTIONAL AMELOBLASTOMA AND HYBRID AMELOBLASTOMA

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ABSTRACT

Review: Ameloblastoma (AM), the most frequently reported tumor originating from odontogenic epithelium, is characterized by a benign but locally invasive behavior with a high risk of recurrence. Hybrid odontogenic tumors (HOTs) consist of two distinct and separable entities growing into single mass clinically as well as microscopically. The exact cause for such an occurrence is not clarified. Epithelial mesenchymal transition (EMT) plays a pivotal role in facilitating the migratory and invasive capabilities of many tumor cell types. E-cadherin functions as an invasion suppressor gene and its expression is decreased in most neoplasms while high osteopontin (OPN) expression is correlated with poor prognosis in different tumors.

Aim of study: The current study aimed to examine the expression of E-Cadherin and OPN in conventional multicystic ameloblastoma (CMAM) and hybrid ameloblastoma (HAM) and correlate their expression with local invasion and aggressive behavior in both lesions.

Material and Methods: Immunohistochemical expression of E cadherin and OPN was evaluated in 7 samples of CMAM cases and 7 samples of HAM.

Results: CMAM expression for E-cadherin and OPN, was significantly different from HAM (P-value ≤ 0.05), the CMAM showed the highest mean value for OPN (17.14±5.11) while HAM showed the higher mean value for E-cadherin expression (13.08±2.15) with statistically non-significant negative relation between E-cadherin and OPN (P-value >0.05) in both lesions.

Conclusion: The higher expression of OPN and lower expression of E-cadherin in CMAM in comparison with HAM indicate that CMAM has local invasion and more aggressive behavior than HAM.

KEY WARDS: ameloblastoma, hybrid tumors, E-cadherin, osteopontin, invasion, EMT

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INTRODUCTION

Odontogenic tumors (OTs) are a diverse group of neoplasms derived from epithelium and mesenchyme that are responsible for teeth formation. Odontogenic tumors are classified by the World Health Organization (WHO) according to their origin whether derived from epithelium or ectomesenchyme components. Benign odontogenic neoplasms originating from epithelial tissues are the most frequently reported. Ameloblastoma (AM) is considered to be the most important neoplasm in this group, representing around 30% of all OTs, followed by Calcifying epithelial odontogenic tumor (CEOT), Adenoameloblastoma and Odontogenic keratocyst (OKC) [1].

Ameloblastoma, is considered the most commonly reported epithelial neoplasms. It is considered a benign neoplasm but has the ability to locally invade the surrounding tissues and usually recurs after treatment. Several researches have been executed trying to explain the invasive activities in AMs. However, the precise biological pathways were not properly clarified [2].

Hybrid neoplasms are composed of two well identified and independent lesions growing into single mass clinically and histologically. The precise reason for their presence was not clarified and this may due to the pluripotent potential of odontogenic epithelium [3].

Examples of hybrid odontogenic tumors (HOTs) are AM with Adenomatoid Odontogenic Tumor (AOT), AM with CEOT, AM with ameloblastic fibroma and AM with glandular odontogenic cyst [4].

Epithelial–mesenchymal transition (EMT) consists of several complicated stages where epithelial cells changes into mesenchymal cells. EMT is the process that mediates how the neoplastic cells migrate, invade the surrounding tissue and plays an important role in formation and growth of AM. The association between EMT and invasiveness of tumors has been reported in various tumors, including meningeal tumors, odontogenic tumors and oral squamous cell carcinoma [5-7].

Several events, such as mutations in many oncogenes (B-type Raf kinase and smoothened) were considered to play an important role in the formation and progression of AM [8].

The cadherin family consists of more than 100 members and categorized into three categories: classical cadherins, non-classical cadherins, and protocadherins. E-Cadherin, one of the most important classical type, is a calcium dependent transmembrane glycoprotein expressed in epithelial cells and able to sustain the integrity of epithelial tissues and polarity. The intracellular domain of E-cadherin is capable of binding to some members of catenin family, including β-catenin, to form intercellular junction complexes. The decreased expression or dysfunction of E-cadherin in tumor cells may be responsible for disruption of epithelium so that tumor cells become able to move from the primary site and acquire migration capability, which could cause the invasiveness and metastasis of neoplastic cells.

In tumors, E-cadherin presence is inversely correlated to the grade in different tumors. Diminished E cadherin expression is associated with progression, invasion and poor prognosis and is correlated with aggressive cancer in many organs as, breast cancer, ovarian carcinomas and glioblastomas [9,10]. So was named "invasion suppressor gene " [11].

Osteopontin (OPN) was recognized as a major sialoprotein in the extracellular matrix of bone. OPN usually exists in several cell types as T-lymphocytes, epithelial cells, osteocytes, macrophages, neoplastic cells and play important role in remodeling processes like inflammation, ischemia-reperfusion, bone resorption and neoplastic progression [12].

Osteopontin was documented to have several roles, where it functions to mediate cell movement, cell survival, and prevention of mineralization. It also regulates the function of immune cells and controls

the phenotype of neoplastic cells. When OPN binds to neoplastic cell membrane receptor CD44v6, this facilitates the neoplastic cell movement and increase the immune adaptation of cells which expressing OPN. OPN induce integrin α_v -mediated signal transduction, this activates osteoclast and increases its osteolytic activity. Ligation of OPN to integrin $\alpha 5\ \beta 3$ on vascular endothelial cells results in the formation of new blood vessels by increasing endothelial cell motility, survival, and lumen formation during angiogenesis [12]. In addition, OPN can interact with HIF2α and impact the AKT1/miR-429/ZEB cascade with subsequent suppression of E-cadherin and activation of EMT [13] so high OPN expression was correlated with invasion and spread of odontogenic lesions into the surrounding bone as reported in OKC, also associated with bad outcome in several neoplasms like non-melanoma skin tumors, prostate and breast carcinoma [14-16].

These pleiotropic roles of OPN in the tumor cascade through activation of EMT and suppression of E-cadherin led to the aim of this study which is to analyze the expression of E-Cadherin and OPN in conventional multicystic ameloblastoma (CMAM) and hybrid ameloblastoma (HAM) and to correlate their expression with local invasion and aggressive behavior in both of them, especially few researches were conducted about these hybrid tumors.

MATERIALS AND METHODS

In the current study, 7 samples of CMAM, and 7 samples of HAM were chosen (4 cases were CMAM + AOT), (3 cases were CMAM + CEOT). All collected from the archive of Oral Pathology Department, National Cancer Institute, Cairo University. Briefly, Immunohistochemical staining was done as follows: wax blocks were cut at four micrometer thickness. Tissue sections were deparaffinized with xylol and rehydrated in grading concentration of alcohol. Tissue sections were placed in citrate buffer before the immunostaining steps. Peroxidase-antiperoxidase process utilizing the biotin-streptavidin system was done, 3% hydrogen peroxide was added to the tissue sections to prevent endogenous peroxidase action.

Primary antibodies E-cadherin (Abcam UK), OPN (Lab Vision, Fermont CA, USA) were applied and then incubated overnight at room temperature. After washing in phosphate buffer saline (PBS), the link antibody was applied, then streptavidin labeling antibody. After rinsing with PBS, diaminobenzidine chromogen was added to the sections then the counterstain. Tissue sections were dehydrated in grading concentration of alcohol, applied in xylol and mounted. All the steps of immunohistochemical quantitative estimation were carried out on photomicrographs captured at a magnification of X40. The images are captured with a camera linked to the microscope and then the images taken are analyzed with the image software (Image J, 1.41a, NIH, USA).

Data was analyzed using Statistical Package for Social Science software computer program version 26 (SPSS, Inc., Chicago, IL, USA). Quantitative data was presented in mean and standard deviation. One way ANOVA(Analysis of variance) followed by post-hoc tukey was used for comparing more than two different groups of parametric data. Pearson's correlation was used to correlate between OPN & E-cadherin . *P* value less than 0.05 was considered statistically significant.

RESULTS

1. Immunohistochemical and statistical Results

A. E-Cadherin

All 7 cases of CMAM demonstrated positive E-cadherin immunoreactivity. More than 75% of stellate reticulum cells showed mainly membranous expression while 75% of the peripheral columnar cells showed negative expression. All 7 cases of HAM (CMAM + AOT), (CMAM and CEOT) showed positive E-cadherin immune expression. The immune reaction was cytoplasmic and membranous in the plexiform strands of ameloblast

cells but in the AOT part the reaction was mainly membranous, while CEOT lesion showed both cytoplasmic and membranous reaction (Fig.1 A,B,C). The One way ANOVA followed by post-hoc Tukey test revealed that CMAM expression for E-cadherin, was significantly different from HAM groups ((P-value ≤ 0.05), the CMAM showed the lowest mean value for E-cadherin (8.04 ± 2.01)). Also (CMAM + AOT) group showed a significant higher E-cadherin expression when compared to the (CMAM and CEOT) lesions (Table 1 (Fig. 2)

B) Osteopontin:

All 7 cases of CMAM demonstrated positive OPN immunoreactivity, 90% of the stellate reticulum cells showed mainly cytoplasmic expression while the peripheral columnar cells showed both membranous and cytoplasmic expression. All 7 cases of HAM (CMAM + AOT), (CMAM and CEOT) revealed positive OPN immune reaction. The immune reaction was mainly cytoplasmic in the plexiform strands of ameloblast cells, while the AOT part revealed mainly membranous expression and the CEOT part showed cytoplasmic reaction (Fig.1 D, E, F) The One way ANOVA followed by post-hoc Tukey test showed that CMAM expression for OPN, was significantly different from HAM groups (P-value ≤ 0.05), the CMAM showed the highest mean value for OPN (17.14±5.11), while there was no significant different between (CMAM + AOT) and (CMAM and CEOT) group (Table 1) (Fig. 3)

2- Correlation between E-cadherin & osteopontin expression in conventional multicystic ameloblastoma and hybrid ameloblastoma groups:

A statistically non-significant negative correlation between E-cadherin and OPN expression (P-value >0.05) between CMAM and HAM groups was observed (Table,2 Fig.4).

TABLE (1): Comparison of E-cadherin & osteopontin between conventional multicystic ameloblastoma, Hybrid ameloblastoma with AOT and Hybrid ameloblastoma with CEOT.

	Conventional multicystic ameloblastoma	**Hybrid ameloblastoma with AOT**	**Hybrid ameloblastoma with CEOT**	P value
Osteopontin	17.14±5.11	10.25±.45[a]	12.37±.88[a]	0.001*
E-cadherin	8.04±2.01	14.19±2.13[a]	11.61±.36[ab]	<0.001*

a: significance vs conventional Ameloblastoma, b: significance vs Hybrid ameloblastoma and AOT

****:significance <0.05***

TABLE (2): Pearson's correlation coefficient for E- cadherin and osteopontin in both conventional multicystic ameloblastoma and hybrid ameloblastoma

		r	**P**
E-cadherin and Osteopontin	All cases	-.521	0.01*
	Conventional multicystic ameloblastoma	.305	.506
	Hybrid ameloblastoma and AOT	0.85	.007*
	Hybrid ameloblastoma and CEOT	-0.74	0.09

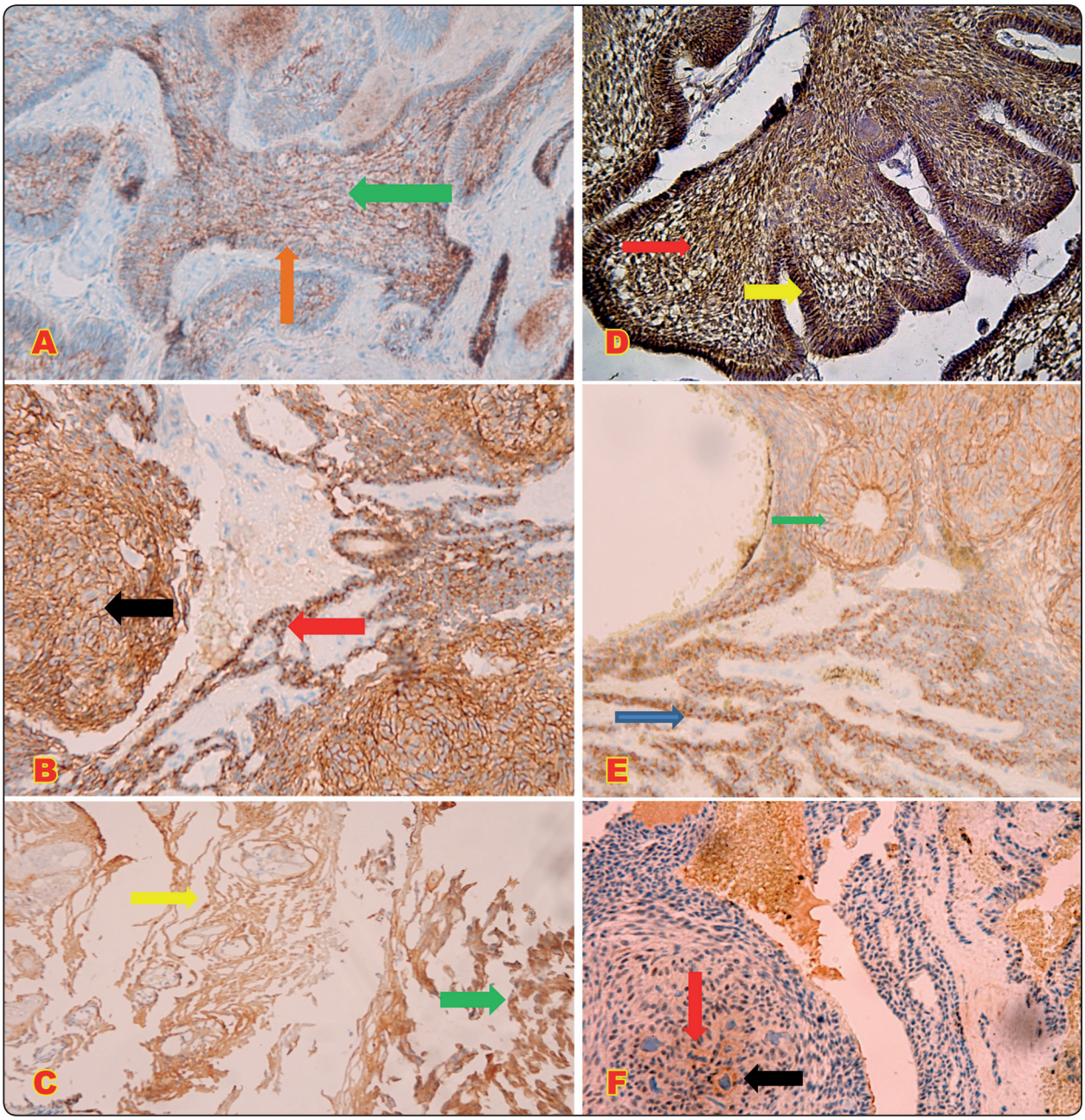

Fig. (1) Photomicrographs of immunohistochemical results of E-cadherin (A,B,C) and osteopontin (D,E,F), in conventional multicystic ameloblastoma and hybrid ameloblastoma lesions. A: Showing membranous reaction of E-cadherin in the stellate reticulum cells of CMAM (green arrow) and negative reaction in the peripheral columnar cells (orange arrow) (Orig. Mag. X40), B: showing the cytoplasmic reaction of E-cadherin in strands of AM (red arrow) and the membranous reaction of AOT part in HAM (black arrow), C: showing the cytoplasmic and membranous reaction of E-cadherin in strands of AM (yellow arrow) and the epithelial cells of CEOT in HAM (green arrow) (Orig. Mag. X20). D: showing the membranous and cytoplasmic expression of OPN in peripheral columnar cells of CMAM (yellow arrow) and the cytoplasmic expression in the stellate reticulum cells (red arrow), E: Showing the cytoplasmic reaction of osteopontin in ameloblastoma strands (blue arrow) in hybrid ameloblastoma, and the membranous reaction (green arrow) of the duct like structures of AOT part F: Showing the cytoplasmic reaction of OPN in epithelial part (red arrow) and the calcification (black arrow) of CEOT in HAM (Orig. Mag. X40).

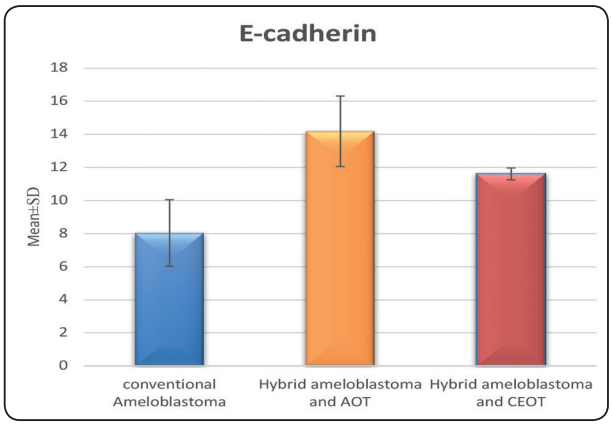

Fig. (2): A bar chart showing the mean values of E-cadherin expression in CMAM and HAM groups

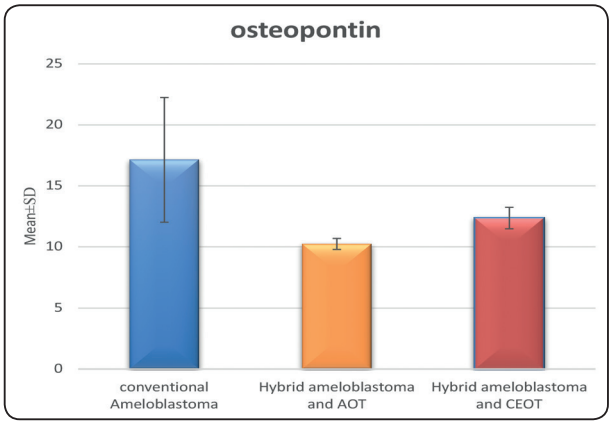

Fig. (3): A bar chart showing the mean values of OPN expression in CMAM and HAM groups

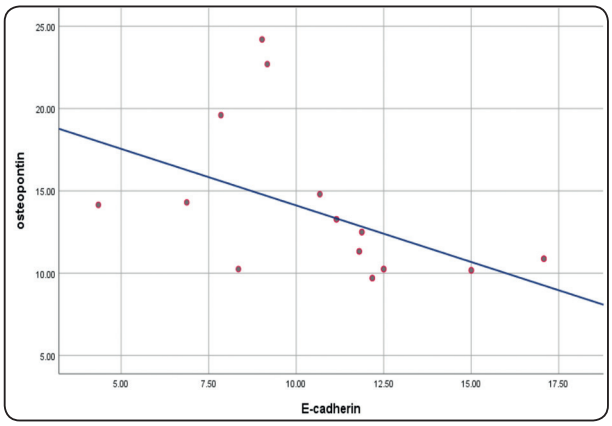

Fig. (4): Scatter diagram representing the correlation between E-cadherin and OPN

DISCUSSION

Ameloblastoma is a locally aggressive odontogenic tumor and recognition of how it infiltrates the surrounding tissues might be beneficial in predicting how these neoplasms will behave [2]. The presence of hybrid neoplasms is debatable and the reason for their occurrence is not fully clarified till now [3].

Since the prognosis of various neoplasms including odontogenic tumors cannot be reliably and accurately predicted on the basis of clinical and histopathologic features only, it is highly desirable to find genetic markers to rely on. These genetic markers could be an objective measure to clarify alterations that might be occurring during the process of tumorigenesis [17]. Taking this into consideration, this study was designed to assess the immunohistochemical expression of E-cadherin and OPN in CMAM and HAM, correlate their expression to each other and to predict their prognostic value in these tumors.

The E- cadherin mediated cell adhesion system is known to function as an "invasive suppressor system" [18-20]. Decreased expression of E-cadherin is usually found with signs of aggressive behavior, more invasive capability, metastasis and tumor recurrence [21].

Concerning the CMAM cases in current study, they showed the lowest significant mean value for E-cadherin (8.04 ± 2.01). More than 75% of stellate reticulum cells showed mainly membranous expression while 75% of the peripheral columnar cells showed negative expression. These findings were similar to those of **Abd Elsamia et al.,** who reported that distribution of immune reaction for E-cadherin was noted on the cell membrane mainly in the central stellate reticulum-like cells than peripheral columnar cells of CMAM [17].

These findings were also in agreement with previous studies which demonstrated that the pattern of E-cadherin immunoreactivity was predominantly on

the membrane at cell-cell borders and the most intense reactivity was noticed in the stellate-reticulum like cells. The intensity decreased in the outer columnar cells, particularly at the invasive front. This indicates that the outer cells of CMAM demonstrate EMT and have the capability of local invasion [22-24].

Another study was conducted by **Qiao et al.,** demonstrate that EMT-asssociated markers such as ß-catenin, Twist, N-cadherin, and vimentin proteins were expressed in high levels in CMAM while E-cadherin was expressed in low levels, indicating that EMT was induced in CMAM tissues in comparison to the tumor-free margins. EMT is very important to mediate how the neoplastic cells migrate and invade the surrounding tissue [25].

Regarding the HAMs cases, all 7 cases of HAM (CMAM + AOT), (CMAM and CEOT) showed the highest significant mean value of E-cadherin expression (14.19±2.13) and (11.61±.36). The higher expression of E-cadherin might reflect the less aggressive behavior in HOTs as it was reported that E-cadherin is a molecule associated with epithelial intercellular adhesion and it's decreased or absent expression is usually associated with invasiveness of neoplasms [26-28]. The cytoplasmic expression of E-cadherin might be explained by failure of E-cadherin to localize to the cell membrane due to genetic or epigenetic alterations in their structure and/or function. Another explanation for cytoplasmic immunopositivity instead of cell membrane reaction of E-cadherin is abnormal tumor-related alteration rather than loss or decreased expression [29, 30].

Concerning the OPN expression in CMAM cases, 90 % of the stellate reticulum cells showed mainly cytoplasmic expression while the peripheral columnar cells showed both membranous and cytoplasmic expression. CMAM expression for OPN, was significantly higher than HAM groups (17.14±5.11). The peritumoral stroma of CMAM also showed OPN immunopositivity. These results were in accordance with **Masloub et al** findings**.** [31].

Wang and Liu [32] demonstrated a similar pattern of OPN immunopositivity in CMAM as they said that OPN protein is most probably produced and released by stellate reticulum like cells, then taken by ameloblast like cells and secreted into the stroma surrounding the tumor nests (transcytosis in ameloblast like cells) in AM. They also stated that it could be possible that ameloblast like cells produce OPN.

As OPN can increase the ability of tumor cells to migrate, spread, invade the surrounding tissues, enhance osteolytic function of osteoclasts, and prevent the immune mediated cytotoxicity against tumor cells, the increased immunoreactivity of OPN in CMAM cells and stromal tissues around the tumor nests, may clarify why CMAM can invade locally through the surrounding tissues and its ability for great amount of bone resorption [32]. Another study of keratocystic odontogenic tumor, which is another highly recurrent odontogenic lesion, both its cystic lining and underlying stroma demonstrated strong OPN immunoreactivity [33]. So the increased immunopositivity for OPN in CMAM might play a role in its high recurrence. The effect of OPN might occur through Binding of OPN to AM tumor cell membrane receptor CD44v6 which in turn enhance tumor cell migration, invasion, and spread and also can elevate the immune adaptation of cells which express OPN [34].

Another study conducted by **Andrade et al.,** showed stronger expression of integrin α5β1 in CMAM than in AOTs when studying them as separate lesions not hybrid. This suggests the participation of this integrin in the local invasiveness of AMs, probably mediated by OPN as it induces integrin α5 mediated signal transduction. Ligation of OPN to osteoclast cell membrane receptor integrin αv can activate the osteoclast and enhance its resorptive activity [35].

Regarding OPN expression in HAM, the 7 cases of HAM (CMAM + AOT), (CMAM and CEOT) revealed the most significantly lower mean values of OPN expression (10.25±.45) and (12.37±.88) respectively**.** The immune reaction was mainly cytoplasmic in the plexiform strands of ameloblast cells, while the AOT part revealed mainly membranous expression and the CEOT part showed cytoplasmic reaction. The decreased expression of OPN in HAM when compared to CMAM could be explained by the possible less aggressive behavior of HOTs which could be supported by previous researches about HOTs which find that only surgical enucleation, was an effective treatment of hybrid lesions and they didn't show any recurrence, or malignant transformation [36-38].

Concerning the hybrid tumor groups in current study the (CMAM + AOT) group showed a higher E-cadherin expression and lower OPN expression when compared to the (CMAM and CEOT) lesions, this could be explained by that CEOT consists of cells that is not differentiated enough to induce any of the odontogenic ectomesenchyme, this make these cells more potent for persistent growth, and explains the true neoplastic potentiality of CEOT and its more aggressive behavior when compared to the highly differentiated cells of AOT which has the ability to induce the formation of dentinoid and enameloid tissues [39-40].

Pearson correlation coefficient test showed a statistically non-significant negative correlation between E-cadherin and OPN expression (P-value >0.05) between CMAM and HAM groups. This could be explained by the fact that the invasive behavior of CMAM is facilitated by OPN biological properties and EMT induced by reduced E-cadherin expression. Another explanation for this negative correlation could be through understanding of the AKT pathway which is a central molecule in cell signaling downstream. The AKT family including AKT1, AKT2 and AKT3 act in different important processes such as cell survival, growth, proliferation, angiogenesis and metabolism. AKT1 and AKT2 have well defined roles in regulating EMT. Nuclear OPN can interact with HIF2α and affect the AKT1/miR-429ZEB cascade with subsequent depletion of AKT1, miR-200s and suppression of E-cadherin [13].

In contrast to our result, **Primali R., et al**[41] reported that hybrid adenoid ameloblastoma presented with higher recurrences, aggressive biological behavior and consider it as a new sub type of CMAM.

Since the long term behavior of hybrid neoplasms remains unknown and enucleation and excision appeared in some cases to cure the hybrid lesion while other cases were treated with more aggressive manner, long term follow up data and additional cases are still needed to substantiate the clinical behavior of these lesions [42, 43]. So further investigations are needed to understand the behavior of HOTs.

CONCLUSION

According to the results of current research, the higher expression of OPN and decreased quantity of E-cadherin in CMAM in comparison with HAM might indicate that CMAM may have more local invasion and aggressive behavior than HAM.

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