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Immunohistochemical Expression of Cortactin, E-Cadherin, and *MDM2* Proteins in Solid Ameloblastoma versus Odontogenic Keratocyst (An immunohistochemical study)

Hanaa M. Abd Elsamia [1*], Nadia A. Radi [2], Eman A. Abo Hager [3], Adel A. Shouman [4]

Codex : 39/1907

azhardentj@azhar.edu.eg

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ABSTRACT

Purpose: The extent of invasion can be analyzed by the expression and production of various genes and proteins by lesional cells. However, current clinical parameters lack the potential to predict the neoplastic behavior in solid ameloblastoma (SAB) and odontogenic keratocyst (OKC). Cortactin, an F-actin binding protein, overexpression has been correlated with advanced clinic pathological stage and poor prognosis in several tumors. E-cadherin belongs to the classical cadherins which. Low E-cadherin expression correlated to aggressive, poorly differentiated, high-grade carcinomas and low patient survival. Human-murine double minute 2 (MDM2), contributes to the promotion of cell growth, survival, invasion, and therapeutic resistance, overexpression of MDM2 has been observed in various human cancers and can contribute to genomic instability, thus, further promoting tumorigenesis. The purpose of this work was to investigate the role of Cortactin, E-Cadherin, MDM2 proteins expression in SAB and OKC and correlate the expression of these markers with the aggressive behavior of these tumors. **Material and method:** 10 case of solid ameloblastoma with its different histologic variants and 10 cases of the keratocystic odontogenic tumor were collected as paraffin embedded blocks. An immunohistochemical investigation using, Cortactin, E-Cadherin, and MDM2 antibodieswere done for all specimens. **Results:** The mean area percent of immunoexpression of Cortactin was greatest in SAB while, the mean area percent of immunoexpression of E-Cadherin, MDM2 were greatest in OKC. **Conculsion:** According to the current study, the absence of any significant differences between AB and OKC indicate the neoplastic and aggressive nature of OKC similar to AB.

KEYWORDS

Cortactin, E-Cadherin, MDM2, Solid Ameloblastoma, Odontogenic Keratocyst

* Paper extracted from PhD thesis titled "Immunohistochemical Analysis of Cortactin, E-Cadherin, and MDM2 Proteins Expression in Selected Benign and Malignant Ectodermal Odontogenic Tumors.

1. Assistant lecturer of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt. email: dr.hanoa@gmail.com
2. Lecturer of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.
3. Professor of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.
4. Professor of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.

INTRODUCTION

Odontogenic tumors consist of a diverse group of lesions because tooth development is a complex procedure in which cells, in different stages of differentiation, contribute to the many phenotypes of histological variety in the odontogenic lesions [1]. The pathogenesis of odontogenic tumors is not clearly distinct and various cell types may take parts in this process. A multitude of odontogenic tumors originates via some deviation from the normal pattern of odontogenesis which reflects their compound multiformity. It is well-known that harmonized collaborations between the epithelial and stromal cells are important for government the growth and clinical manners of pathoses[2-4]. Ameloblastoma (AB) is a benign epithelial odontogenic neoplasm of the jaw with an insidious growth pattern. It is frequently aggressive and destructive, able to achieve great size, corrode bone and invade adjacent tissues [5,6]. Odontogenic keratocyst (OKC) is benign uni or multicystic, intrabony cyst of odontogenic source, with unique distinguishing lining of the parakeratinized stratified squamous epithelium. Due to its histopathological variant characteristics, specific clinical course, high recurrence rate, high rate of proliferative activity, invasive behavior and relation to the nevoid basal cell carcinoma syndrome all these features reflect the neoplastic nature of these lesions. The invasion extent can be investigated by the expression of different genes proteins by tumoral cells. However, current clinical parameters lack the potential to anticipate the neoplastic behavior in both SAB and OKC [7-9].

Cortactin is an F-actin binding protein (filament) originally known as a substrate for Src family kinases (SFK) [10]**.** Originally, little was known about its function, except that it bounds to actin filaments leading to enhancement of cell motility and loss of cell-cell adhesion this has been accomplished through its involvement in all steps of the invadosome lifecycle, from assembly, maturation, proteolytic activity, and disassembly. Cortactin overexpression was found in different cancers such as head and neck squamous carcinoma, breast, hepatocellular carcinoma, and bladder cancer, and linked with reduced patientprognosis and decline survival. Its overexpression has been corresponding to advanced clinic stage and poor out come in several tumors [11-13].

The cadherin family consist of more than 100 members and divided into three subfamilies: classical cadherins, nonclassical cadherins, and protocadherins. Of these, classical cadherins are the greatest widely studied and E-cadherin belongs to the classical cadherins mediating intercellular adhesion at the level of epithelia so maintains the structural and functional integrity of epithelial tissues [14]. E-cadherin expression is inversely correlated to the grade of differentiation in different cancers [15,16]. On the other hand, E-cadherin is related to aggressive cancer in many organs as, breast cancer, ovarian carcinomas, and glioblastomas, where E-cadherin is linked to decline survival and correlates with enhanced invasiveness [17-20].

The murine double minute 2 (MDM2) is an oncoprotein which share in the promotion of cell proliferation, survival, invasion, and treatment resistance. MDM2 is a negative regulator of the p53 tumor suppressor protein through binding to the transcriptional initiation domain of p53 leading to proteasome-mediated degradation. High expression of MDM2 lead to genomic instability, thus, promoting tumorigenesis [21]. Increase expression of MDM2 has been detected in many human malignancies involving melanoma, breast carcinoma, glioblastoma, leukemia [22]. Overexpression of MDM2 in ameloblastomas indicates that an increased production or decreased breakdown of protein leads to disturbance in growth regulation [23]. To date, no appropriate immunohistochemical marker is presented to estimate the aggressiveness of both SAB and OKC, therefore, the objective of this study is to assess the association of both Cortactin, E-Cadherin, and MDM2 expression in both SAB and OKC and analyzing their correlation with the biological behavior of these neoplasms by means of the immunohistochemical technique.

MATERIALS AND METHODS

Case selection

The specimens for this study were retrieved as paraffin-embedded blocks from the archives of Oral Pathology Department, Faculty of Dentistry, Alexandria University. The specimens were divided into two groups according to WHO classification (10 cases of SAB with its different histologic variants and 10 cases of the OKC.

Histological analysis

Using H&E for reevaluation of the abovementioned cases was carried out to confirm their diagnosis, and reclassifying them according to WHO [24].

Immunohistochemical analysis

Sections of 4 μm thickness were mounted on electrically charged slides and deparaffinized. To block endogenous peroxidase activity, the samples were subjected to 3% hydrogen peroxide solution for 5 minutes. They were subjected to heat-prompted antigen retrieval procedure using citrate buffer (10mM, pH 6.0) in a pressure cooker for 2 min. Consequently, the tissue sections were incubated with primary monoclonal antibodies according to the manufacturer's directions using Cortactin, E-Cadherin, and MDM2 mouse monoclonal antibodies (Clone A-4 Cat.No.SC-55578; Clone NCH-38, and Clone 1B10). Followed by 45 min in secondary antibody (rabbit antibody against mouse immunoglobulins) (DAKO, Denmark). It was then immersed in streptavidin-biotin peroxidase conjugate (DAKO) and incubated for 10 min with 3, 3-diaminobenzidine chromogen (DAKO). Mayer's hematoxylin was used as counter-stain. All the steps were carried out at room temperature and after each step the sections were rinsed with PBS, pH 7.2[25].

Histomorphometric analysis

Immunoreactivity, for Cortactin, E-Cadherin, and MDM2; were evaluated by assessing the area percent of positive immunostained cells in relation to the area inspected in each field using image analyzer computer system (Leica, Germany) at Oral and Dental Pathology Department, Faculty of Dental Medicine for Girls, Al Azhar University. Using the color detection, areas of positive immunostaining were covered by a blue binary color. Ten fields per each tissue section of each patient were sequentially taken, to be histomorphometrically assessed. Mean values were then gotten for each tissue section.

Statistical analysis:

Data were represented as means and standard deviation (SD) values, ANOVA test used to compare means of more than two groups. Tukey-Kramer multiple comparisons were used in the procedure of pairwise comparisons between the groups when the ANOVA test is significant. The P value is significant if less than or equal to 0.05 ($P \leq 0.05$). Statistical analysis was achieved by means of instate graph pad version 3.10 and Microsoft® excel 2007.

RESULTS

Histopathological findings:

SAB has five different histopathological variants. They are **follicular** ameloblastoma showed multiple nests of odontogenic epithelium. The outer cells are tall columnar with reverse polarity resembling ameloblasts and central stellate reticulum like cells surrounded by connective tissue stroma. These islands sometimes surrounded by juxta follicular hyalinized zone of fibrous connective tissue**.** Cystic degeneration in the central stellate reticulum- like cells of the follicles resulted in the formation of microcysts was observed in these islands (Fig. 1; A), while in acanthomatous variant the neoplastic island

showed central stellate reticulum like cells with formation of squamous cells with eosinophilic cytoplasm (Fig. 1; B), **basal cell ameloblastoma** composed of islands of uniform compacted dark stained cuboidal basaloid cells surrounded by juxtaepithelial hyalinized homogenous fibrous tissue band(Fig. C), **granular cell ameloblastoma**, showed tumor islands with peripheral columnar cells surrounding central large cells with granular eosinophilic cytoplasm and peripheral nuclei (Fig. 2;M) and **plexiform ameloblastoma** showed anastomosing cords of ameloblastoma epithelium, the peripheral cells are cuboidal and the central cells are few loosely arranged stellate reticulum like cells within degenerated connective tissue stroma (Fig. 2;N).

OKC showed cystic lumen lined by thin uniform epithelium without rete pegs. It surrounded by connective tissue wall containing daughter cyst. There is an area of detachment of a portion of epithelial lining from the fibrous connective tissue wall (Fig. 2; O).

Immunohistochemical findings:

Cortactin immunostaining was seen in the cytoplasm and/or cell membrane of the outer basal and stellate-reticulum like cells of the neoplastic islands of all histopathological variants of solid ameloblastoma while it was negative in central squamous cells of acanthomatous type (Fig. 1; D, E, F) (Fig. 2; P, Q). While in OKC cortactin immunostaining was seen in the cytoplasm and/or cell membrane of the entire epithelial lining. Few connective tissue fibroblasts showed positive immunostaining (Fig. 2; R).

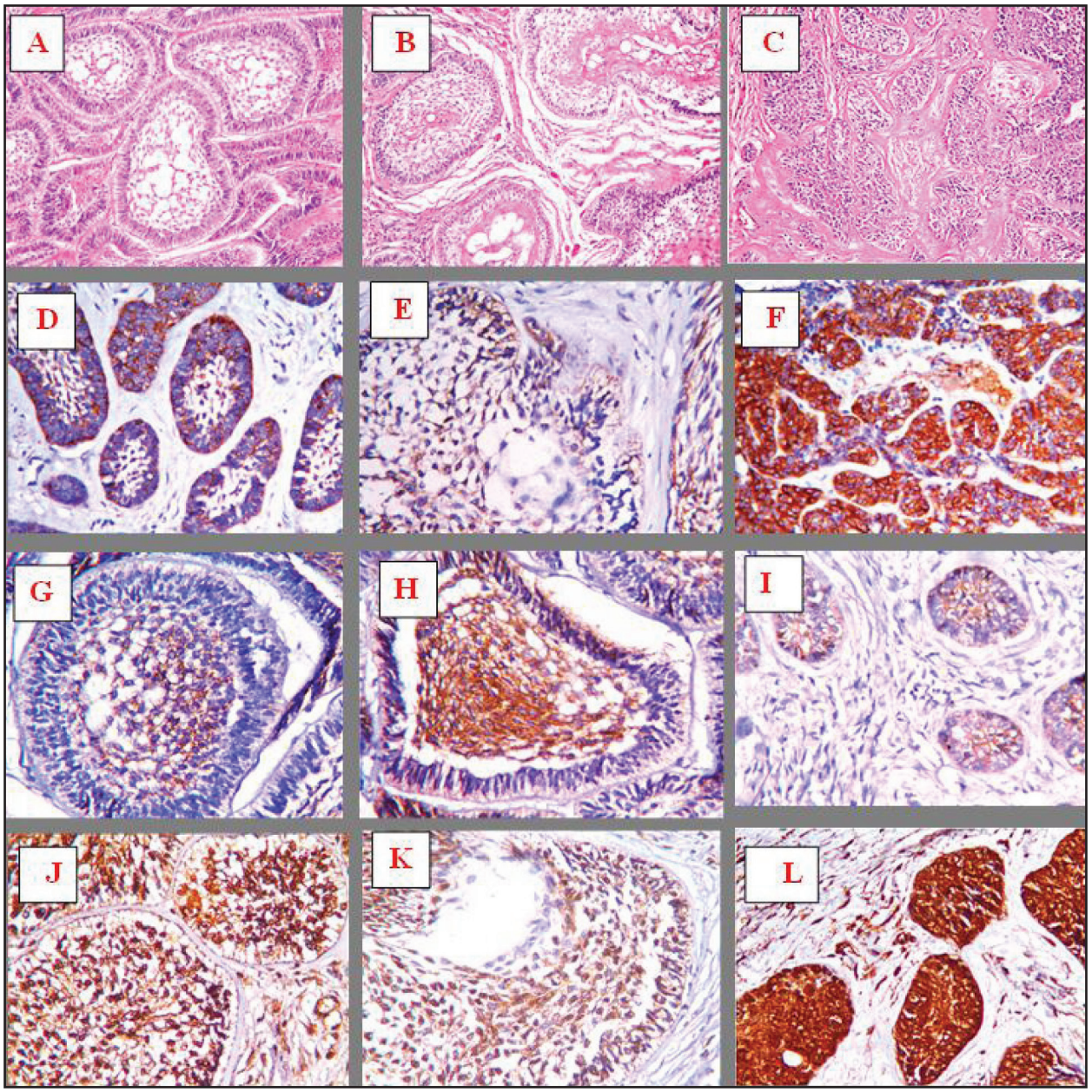
E-cadherin immunostaining was seen on the cell membrane and less frequently cytoplasmic chiefly of central stellate– reticulum like cells. Few outer cells of the neoplastic nests may show positive immunostaining in all histopathological variants of solid ameloblastoma except basal cell ameloblastoma, immunostaining was membranes of the basaloid cells of the neoplastic island (Fig. 1; G, H, I) (Fig.2; S, T). While in OKC E-cadherin immunostaining was seen on the cell membrane of suprabasal layers, while it was negative in the basal cell layer and parakeratinized surface (Fig. 2; U).

MDM2 immunostaining was seen in the nucleus and cytoplasm of cells of the neoplastic islands including all histopathological variants of solid ameloblastoma while it was negative in the central squamous cells of acanthomatous type (Fig. 1; J, K, L) (Fig. 2; V, W). While in OKC, MDM2 immunostaining was seen on the nucleus and cytoplasm of all layers of epithelial lining. Some connective tissue fibroblasts showed positive nuclear immunostaining (Fig. 2; X).

Statistical Results

Comparison between SAM and OKC

The statistical difference between solid ameloblastoma and odontogenic Keratocyst was not significant considering the three markers as showed in (Table. 1,2, 3& Fig. 3,4,5).



Figure(1) Follicular ameloblastoma showed multiple islands of odontogenic epithelial cells **A,** acanthomatous variant, the neoplastic island showed central stellate reticulum like cells with formation of squamous cells **B,** basal cell ameloblastoma composed of islands of uniform compacted dark stained cuboidal basaloid cells **C, (A-C, H&E X 200).** Cortactin immunostaining was seen on cytoplasm and/or cell membrane of follicular, acanthomatous, and basal cell variants of AB **(D-F, cortactin, X200).** E-cadherin immunostaining was seen on cell membrane and cytoplasm mainly of central stellate- reticulum like cells of follicular and acanthomatous, while in basal cell ameloblastoma, showed membronus expression of the basaloid cells **(G-I, E-cadherin, X 200)**. MDM2 immunostaining was seen on the nucleus and cytoplasm of peripheral cells and central stellate reticulum like cells of follicular, acanthomatous, and basal cell variants of AB, while it was negative in the central squamous cells of acanthomatous type **(J-L, MDM2, X 200)**.

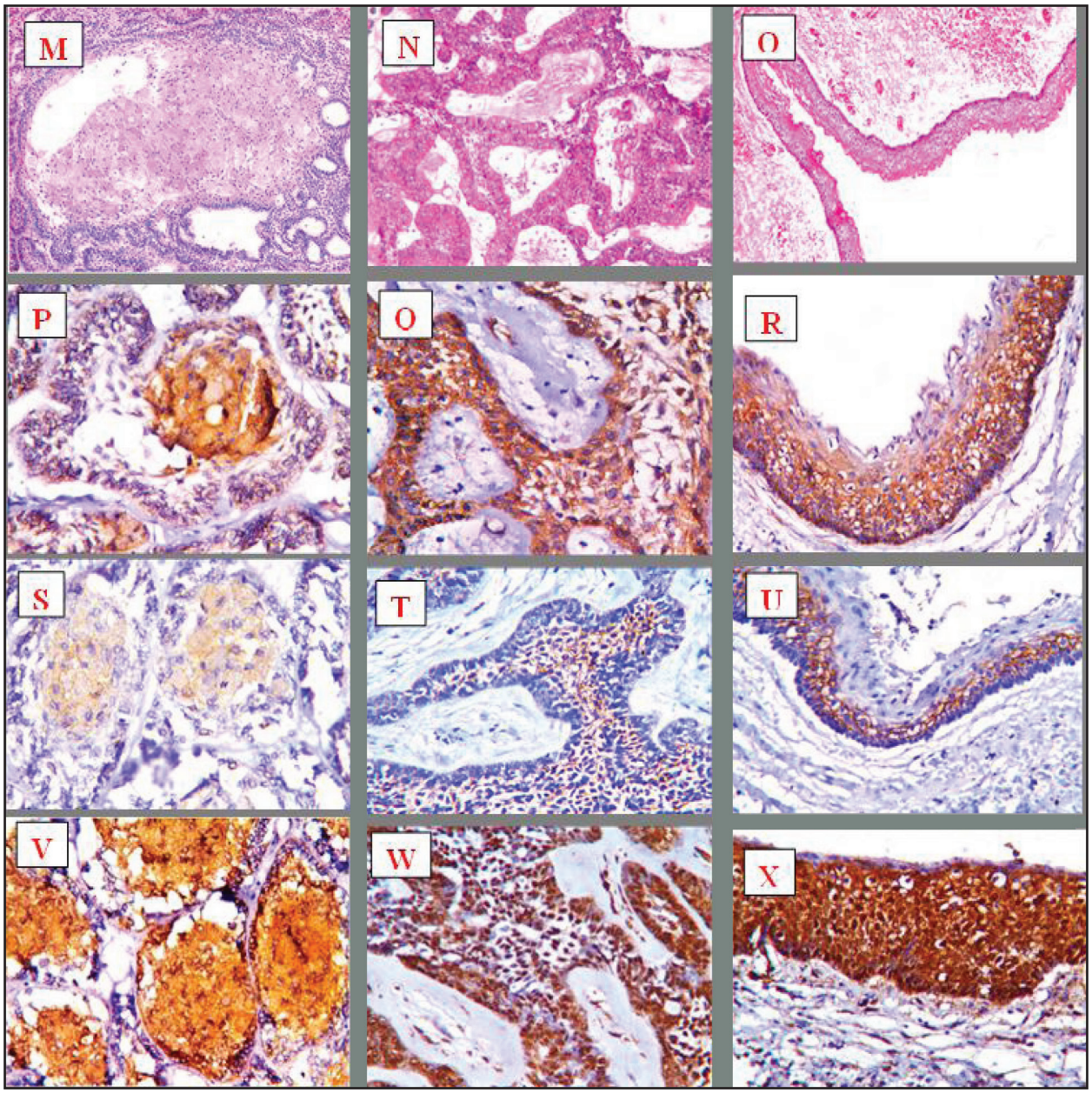


Figure (2) Granular cell ameloblastoma, showed tumor islands with peripheral columnar cells surrounding central large cells with granular eosinophilic cytoplasm and peripheral nuclei **M,** plexiform ameloblastoma showed anastomosing cords of ameloblastoma epithelium **N**, OKC, showed cystic lumen lined by thin uniform epithelium without rete pegs **O (N-O, H&E X 200),** Cortactin immunostaining was seen on cytoplasm and/or cell membrane of granular **P**, and plexiform **Q** variants of AB. OKC cortactin immunostaining was seen on cytoplasm and/or cell membrane of all layers of epithelial linning **R**. **(P-R, cortactin, X200).).** E-cadherin immunostaining was seen on cell membrane and cytoplasm mostly of central stellate– reticulum like cells of granular **S**, and plexiform **T**, variants of AB while in OKC E-cadherin immunostaining was seen on cell membrane of supra basal layers, but it was negative in the basal cell layer and parakeratinized surface **U**, **(S-U, E-cadherin, X 200)**. **MDM2** immunostaining was seen on the nucleus and cytoplasm of peripheral cells and central stellate reticulum like cells of neoplastic islands of granular **V**, and plexiform **W**, variants of AB. While in OKC, MDM2 immunostaining was seen on the nucleus and cytoplasm of all layers of epithelial lining **X**, **(V-X, MDM2, X 200).**

Table (1) *Comparison between cortactin area % in SAM&OKC*

Tumor type	Mean	SD	Median	Minimum	Maximum	
Solid ameloblastoma	37.47[B]	5.80	36.01	29.91	44.13	<0.001*
Keratocystic odontogenic tumor	37.22[B]	2.90	38.41	33.21	40.42	

**: Significant at P ≤ 0.05*

Table (2) *Comparison between E-cadherin area % in SAM&OKC*

Tumor type	Mean	SD	Median	Minimum	Maximum	
Solid ameloblastoma	14.14[B]	1.93	13.99	11.72	16.56	<0.001*
Keratocystic odontogenic tumor	14.93[B]	2.93	14.58	11.30	19.78	

**: Significant at P ≤ 0.05*

Table (3) *Comparison between MDM2 area % in SAM&OKC*

Tumor type	Mean	SD	Median	Minimum	Maximum	
Solid ameloblastoma	39.91[B]	1.90	39.81	37.59	42.10	<0.001*
Keratocystic odontogenic tumor	40.94[B]	3.63	40.97	35.81	45.76	

**: Significant at P ≤ 0.05*

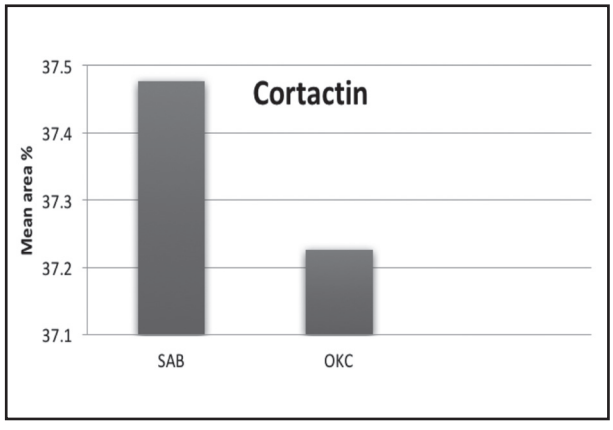


Figure (3) Bar chart representing mean values for cortactin area % in SAB and OKC.

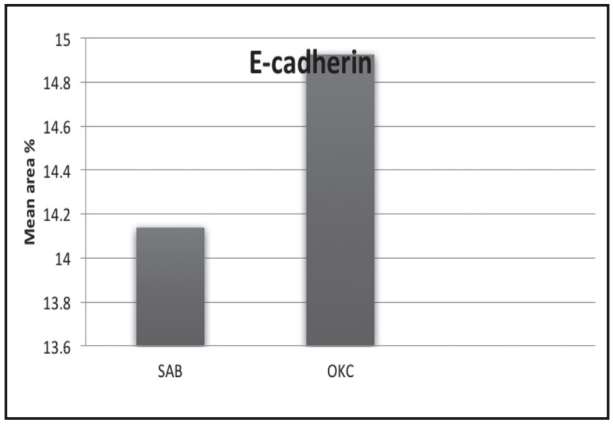


Figure (4) Bar chart representing mean values for E-cadherin area % in SAB and OKC.

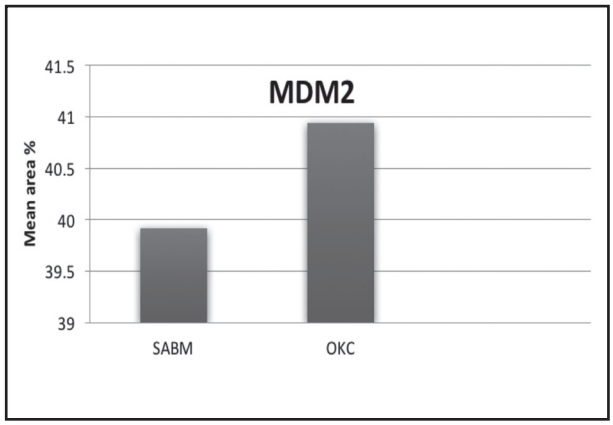


Figure (5) Bar chart representing mean values for MDM2 area % in SAB and OKC

DISCUSSION

Since the prognosis of various neoplasms including odontogenic tumors cannot be reliably and precisely predicted on the basis of clinical and histopathologic features only, it is highly desirable to find genetic markers to rely on. The last could be an objective measure to gain insight into alterations that might be occurring during the process of tumorigenesis. So, the existing study was designed to assess the immunohistochemical expression of cortactin, E-cadherin and MDM2 in SAB and OKC, correlate their expression to each other and to predict their prognostic value in these tumors.

Regarding SAB, cortactin immunostaining was detected in the peripheral and central stellate cells of the tumoral epithelial nests while it was negative for squamous area and keratin in the acanthomatous subtype. These results were in accordance with other studies that showed acanthomatous epithelial tumors were non-reactive for invadopodia proteins and they explained this as tumor areas with strong invadopodia protein expression would related to sites of dynamic actin remodeling while non-expression mean a shift to dormancy [26,27]. Also, cortactin immunolocalization was cytoplasmic and membranous. This finding was explained as cortactin directed to the cell membrane to induce invadopodia assembly. In addition, cortactin could attach to actin-related molecules complexes that might act as bridges in the early steps of invadopodia development**.** It was revealed that local invasiveness of ameloblastoma is rely on the migrant potential of tumor cells as defined by their allocation of cortactin and other invadopodia proteins. It was suggested that cell migration and extracellular matrix destruction affect ameloblastoma behavior and that these cellular activities are concomitant with cortactin-intermediated invadopodia development and membrane-type 1 MMP (MT-MMP) enrolment[28-30].

Regarding OKC, cortactin immunostaining was seen as cytoplasmic and membranous localization in all layers of OKC. These results were in accordance with other studies [31,32]. It was reported that OKC is a multicystic neoplasm, which could show daughter cysts that occur sporadically as invadopodia may share in this process due to its capability to facilitate local invasion. Invadopodia pericellular activity possibly lead to separation of one or more tumor cells from the primary cyst, result in creation of daughter cyst and this occurrence supported by increase expression of invadopodia-associated proteins like cortactin and MT1-MMP in OKC [28]**.**

In the present study, the distributions of positive immune reaction were variable among the studied tumors, where there no statistically significant difference between SAB and OKC. This finding was in agreement with another result that revealed expression of podoplanin which modulate the actin cytoskeleton and act as a mediator in the pathway of cellular invasion was expressed in OKC and ameloblastomas with no significant difference [33]. Invadopodia proteins have been recognized as a principle element of the crosstalk between tumor cells and their surrounding microenvironment. Significant to their role in promoting tumor progress, invasion and metastases are their capacity to orchestrate actin cytoskeleton remodeling in tumoral as well as stromal parts. Tumor cells can affect the contractile property of stromal cells. Enlarged stromal cell contractility result in high matrix rigidity and this has tumor- enhancing effects. It is likely that the noticed various distribution

form for cortactin in ameloblastoma epithelial and stromal cells could reflect the various steps of actin turnover. It was found that although SAB and OKC exhibit slow growth; however, they have local invasive manners and high rate of recurrence if not treated with more aggressive actions [27, 34-36].

Regarding SAB, in the current study, the distributions of immune reaction for E-cadherin were noted on the cell membrane mainly in the central stellate reticulum-like cells than peripheral columnar cells of ameloblastoma. These results are agreed with previous results 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, especially at the invasive head. This indicates that the outer cells of ameloblastoma display EMT and have the ability of local invasion [16, 37, 38]. It was reported that the immunoreaction for E-cadherin in central parts of tumor nests in SAB that morphologically like to the stellate reticulum of the enamel organ propose that this neoplastic epithelial element preserve the characteristics of cytodifferentiation of the odontogenic epithelium [39]. Other study revealed the highest E-cadherin expression in the stellate reticulum like cells, suggested a possible higher level of this protein at certain areas where it enhance the adhesion between the distant cells in this organization regarded as a compensatory phenomenon that is an attempt to maintain normal cellular architecture [40].

On the other side, it was found that E-cadherin reaction was weak or disappear in the squamous metaplastic areas and keratinized areas of acanthomatous ameloblastomas, due to end differentiation of the tumor cells. Also, in granular ameloblastoma, E-cadherin expression was weak cytoplasmic and membranous in clusters of granular cells as these cells have high apoptotic reaction in comparison with other types of ameloblastoma. These results might be due to end differentiation of the tumor cells such as degenerative and/or maturation changes and were not consider progression or malignant potential of the tumors [37].

Concerning OKC, in the current study, the distributions of positive immune reaction for E-cadherin were observed on the cell membrane mainly of suprabasal layers. These results were in accordance with another study [39] described the higher reaction of E-cadherin in the suprabasal layers of the epithelial lining of OKC, representing a high degree of cell adhesion between epithelial cells of these layers of the tumor. While, a moderate to high-grade loss of E-cadherin staining in all tissue samples of OKC was found and explained as a down-regulation of β-catenin and E-cadherin in basal and luminal parakeratinized cell layers of OKC, indicating an invasive potential [41].

In the current study, the mean value of area percent of E-cadherin immunostaining was higher in OKC than SAB with no statistically significant difference between them. These results are in line with other's results [42] confirmed that adhesion molecules as (CD166) were expressed in ameloblastoma and OKC with no statistically significant difference between them. This could be explained by decreased to the negative reaction of E-cadherin in peripheral basal cells of both lesions where they express EMT phenotype and have the potential of focal invasion. The E-cadherin complex redistributation from the cell membrane to cytoplasm explained by failure of E-cadherin and the catenins to localize to the cell membrane and/or bind the cytoskeleton regardless their plenty may be due to genetic or epigenetic alterations in their structure and/or function. Another explanation for cytoplasmic expression pattern instead of cell membrane reaction of E-cadherin is abnormal tumor-related alteration rather than loss or reduction of expression [43].

Regarding SAB, the positivity for MDM2 was observed in nucleus and cytoplasm of the outer and central stellate reticulum like cells. These results were in agreement with a previous study [23] representing the expression of MDM2 was almost confined to nuclei except on occasions

cytoplasmic staining was observed**.** The MDM2 positive nuclei were found predominantly in peripheral ameloblast-like cells and it was also seen in central stellate reticulum- like cells suggesting that the peripheral cells have an anti-apoptotic and proliferative phenotype than the central cells. This also, in accordance with other study stated that the proliferative activity of ameloblastomas is nearly limited to the outer layer of the epithelial islands [(44)].

Regarding OKC, it showed positive cytoplasmic and nuclear immunostaining of MDM2 in all layers of epithelial lining suggesting a neoplastic origin for OKC and distinct biological behavior than the other non-neoplastic odontogenic cysts, as for proliferation, apoptosis and differentiation processes [(45)].

In the present study, the mean area percent of MDM2 immunostaining was higher in OKC than SAB without significant difference between them. These results agreed with previous study found that the reaction of MDM2 was high in ameloblastoma and OKC without significant difference **[(45)]**. It was suggested that increased reaction of MDM2 proteins plays a role in pathogenesis and tumor development of SAB and OKC, and this amplified expression give an explanatory mechanism for the likenesses in the biologic behavior of the two lesions and their activity in the same way**.** Moreover, these results go with other outcomes suggested that OKC and SAB have similar proliferation indices in the basal compartment whereas, in the suprabasal compartment, OKC has a higher proliferative capacity. This calls for an aggressive and more radical treatment for OKC too than just a simple enucleation as is usually performed [(46)].

CONCLUSION

From the previous results, we can conclude that Cortactin can be used as a potential invasive marker to differentiate between aggressive and nonaggressive odontogenic tumors as well as the absence of any significant differences between SAB and OKC indicate the aggressive nature of OKC.

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