EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 69, 1129:1138, APRIL, 2023
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 30-01-2023 • Accept Date : 18-02-2023 • Available online: 01-04-2023 • DOI : 10.21608/edj.2023.190709.2421

DOUBLE IMMUNOHISTOCHEMICAL ANALYSIS OF PCNA AND FAK EXPRESSION IN SELECTED ODONTOGENIC LESIONS

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ABSTRACT

Background: Odontogenic neoplasms and cysts have diverse biological behaviors, ranging from indolence to aggression along a spectrum. The proliferating cell nuclear antigen (PCNA) is an antigen involved in DNA synthesis and proliferation. Focal adhesion kinase (FAK) is a cytoplasmic molecule associated with cellular signaling, growth, and invasion. PCNA and FAK markers have been shown to be involved in neoplastic proliferation, invasion, and migration. Hence, an immunohistochemical assay of PCNA and FAK can be used as a predictive tool for the level of aggressive behavior of odontogenic lesions.

Methods: The current study was conducted to evaluate the expressions of PCNA and FAK. A double immunohistochemical technique for conventional ameloblastoma (AB), unicystic AB, calcifying epithelial odontogenic tumor (CEOT), and glandular odontogenic cyst (GOC) was used to evaluate the role of both markers in assessments of the aggressiveness of selected odontogenic lesions. All formalin-fixed paraffin-embedded blocks (n = 10) for each studied group were double PCNA and FAK immunostained and then assessed using a transmission light microscope and an image-analyzer computer system. Statistical analysis was performed with a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test.

Results: All study groups showed nuclear immunoreactivity of PCNA and cytoplasmic immunoreactivity for FAK. The greatest mean nuclear count of PCNA and the greatest mean area percent for FAK were both recorded in the aggressive lesions; i.e., the CEOT group and conventional AB group. The non-aggressive unicystic AB group had a lower mean nuclear count for PCNA and showed the lowest mean area percent for FAK.

Conclusions: In conclusion, PCNA and FAK immunoexpression profiles may have a strong correlation with the aggressive nature of AB, CEOT, and GOC. Hence, PCNA and FAK markers could aid in their routine examination, treatment planning, and prognosis.

KEYWORDS: PCNA, FAK, Ameloblastoma, Unicystic Ameloblastoma, Calcifying Epithelial Odontogenic Tumor, Glandular Odontogenic Cyst, Double Immunohistochemistry.

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INTRODUCTION

Odontogenic lesions are a diverse group of neoplasms and cysts, arising exclusively in the maxillofacial region from elements of the odontogenic apparatus [1,2]. Benign odontogenic tumors and cysts encompass a spectrum of biological behavior, ranging from indolent encapsulated lesions to highly aggressive lesions with local infiltration and high recurrence rates [3]. Incidence of the odontogenic tumors is relatively rare, with discrepancies in their epidemiology [4].

Ameloblastoma (AB), a benign epithelial odontogenic tumor, originates from the dental organ, odontogenic remnants, cyst lining, or basal cells of the oral epithelium [5]. AB is considered one of the most frequent odontogenic lesions, accounting for 1%–3% of all gnathic cysts and tumors and 10% of odontogenic neoplasms [6]. This tumor usually manifests as a slow-growing, asymptomatic swelling. Nevertheless, despite its benign nature, it is a highly aggressive lesion characterized by local invasion, destruction of jawbones if left untreated, and a high recurrence rate [7]. According to the World Health Organization, an updated classification of AB includes: i) AB (conventional), ii) unicystic AB, and iii) extraosseous/peripheral types, because they have significant clinicopathologic/biologic differences. Thereby, a proper clinical-radiologic-pathologic correlation implies the type of AB and consequent proper management. Radiographically, conventional AB frequently exhibits well-defined multilocular radiolucency with a typical soap bubble appearance and less frequently has a unilocular appearance [8]. The histopathology of AB is variable. It mainly comprises follicular and plexiform histological patterns, and less commonly acanthomatous, granular cell, clear cell, and desmoplastic patterns, without any histological impact on tumor prognoses [8,9]. Unlike conventional ABs, the unicystic type is characterized by the following: a) tumor growth limited to the lumen with a very thin cystic lining and displays of only three varieties: luminal, intraluminal, and mural (otherwise it shares all of the classic histopathologic features of an AB), b) it has a unilocular radiolucent pattern, and c) it is considered less aggressive and less invasive; hence it responds effectively when treated with a conservative approach [10].

A calcifying epithelial odontogenic tumor (CEOT), also known as a Pindborg's tumor, is an epithelial odontogenic neoplasm. CEOTs comprise less than one percent of all odontogenic neoplasms. Although they are benign, this tumor exhibits aggressiveness and invasiveness in addition to cortical bone expansion and infiltration into the surrounding tissues [11]. Radiographically, CEOTs display unilocular or multilocular radiolucency. When aging, the lesion exhibits a distinctive feature of an expansile radiolucency intermixed with opacities, called a "snow-driven pattern." Interestingly, this type of tumor has distinct histopathology comprising sheet-like masses of polyhedral neoplastic cells with prominent intercellular bridges and nuclear pleomorphisms, amyloid proteins, and characteristic concentric calcified deposits, known as "Liesegang rings" [12].

A glandular odontogenic cyst (GOC) is an odontogenic cyst displaying epithelial glandular differentiation. Recently, GOCs have been considered a familiar entity that commonly presents as a slowly growing mandibular lesion that has a high tendency for recurrence. Radiographs reveal well-defined unilocular or multilocular radiolucency with possible scalloped borders and a particular ability to cross the midline. The epithelial lining of this cyst exhibits numerous histologic parameters, including an epithelial lining of variable thickness, epithelial spheres, multiple compartments, intraepithelial microcysts, apocrine metaplasia, mucous cells, clear (vacuolated) cells, hobnail (cuboidal) cells, tufting (papillary projections), and cilia. To make an accurate diagnosis, 7 out of 10 specific microscopic parameters are required. Interventions range from simple enucleation to resection for large or multilocular lesions [13,14].

Although hematoxylin and eosin (H&E) staining constitutes the standard pathological diagnostic technique, immunohistochemistry is a well-established tool of significant value, particularly when assessing further prognostic factors of different tumors [15]. Proliferating cell nuclear antigen (PCNA), a cell cycle sliding ring-shaped protein, acts as an antigen involved in DNA synthesis and repair as well. PCNA protein peaks in expression during the S-phase of the cell cycle. Therefore, PCNA is broadly considered a marker of cellular proliferation in neoplasms[16]. Also, as it is implicated in neoplastic activity and invasiveness, PCNA is useful as a prognostic marker[17]. Several studies have evaluated PCNA expression in odontogenic lesions where it is overexpressed in lesions with an aggressive nature in comparison to indolent lesions exhibiting minimal recurrence and invasiveness, showing a significant difference [18,19]. Focal adhesion kinase (FAK) is a cytoplasmic molecule that is closely associated with the cell membrane. FAK belongs to the tyrosine kinase family and is an essential mediator of signal transduction pathways and receptor signaling [20]. Upon its activation, subsequent signaling cascades in many cell processes are triggered, including survival signaling, growth, angiogenesis, migration, and invasion [21]. Recent studies have pointed to strong FAK expression in aggressive and invasive odontogenic lesions compared to weak expression in non-aggressive ones, suggesting the possible role of FAK in the aggressive behavior of some odontogenic lesions [22,23].

The current study was carried out to investigate various immunohistochemical expression of PCNA and FAK in odontogenic lesions (conventional AB, unicystic AB, CEOT, and GOC), utilizing a double immunostaining technique that may be helpful to evaluate the role of both markers in the aggressiveness of the selected odontogenic lesions.

MATERIALS AND METHODS

Tissue samples

The present study was a comparative *in vitro* study that utilized formalin-fixed paraffin-embedded specimens. The tissue samples were sourced from the archived files of the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Cairo University, Egypt. Forty specimens of selected odontogenic lesions were retrieved (10 cases of conventional AB, 10 cases of unicystic AB, 10 cases of CEOT, and 10 cases of GOC). The H&E-stained cases were reviewed by two pathologists in addition to the authors to confirm the appropriate diagnosis. Then, the tissue sections were prepared for immunohistochemical staining.

Immunohistochemical staining

Sectioning of all formalin-fixed paraffin-embedded blocks (n = 10) for each studied group was applied with a 4 μm thickness. Then sections were deparaffinized and prepared for double immunohistochemical staining with both anti-PCNA (Dako Agilent Technologies, Inc., CA, USA) and anti-FAK antibodies (Novus Biologicals, CO, USA). The process of double immunohistochemical staining was done following the manufacturer's instructions at a dilution of 1:100 using an Automated Stainer (Ventana Bench-Mark Auto-Stainer, AZ, USA) at the Department of Pathology, National Cancer Institute, Cairo University, Egypt. Regarding the positive labeling for immunoreactions, nuclear PCNA staining was visualized using a diaminobenzidine chromogen kit (Thermo Fisher Scientific, Inc., MO, USA), whereas for FAK, an aminoethyl carbazole chromogen kit (Novus Biologicals, CO, USA) was used to detect a red cytoplasmic color in an unstained background. Additionally, formalin-fixed paraffin-embedded "B-cell lymphoma" and "human spleen tissue" samples were used as a positive control for PCNA and FAK, respectively.

Immunohistochemical assessment

In an unstained background, positive immunoreactions to PCNA were identified by a brown nuclear color, whereas positive immunoreactions to FAK were visualized by a red cytoplasmic color presentation. The most homogenous portions of the reaction were evaluated using both a transmission light microscope and an image-analyzer computer system.

Transmission light microscopy: The PCNA and FAK immunostained sections were assessed with low and high-power fields using a transmission light microscope (Leica model DM LB2, Switzerland).

Image analysis computer system: An image-analyzer system, employing Leica Quin 500* software (Leica Microsystems LTD., Switzerland), was applied for automated measurement of cell counts of PCNA-positive immunoexpression as well as the area percent of FAK-positive immunoexpression in five fields per case, using a standard frame area of 248 × 103 μm^2 at 200 × magnification. For each of the groups tested, the mean cell count and mean area percent values were determined for PCNA and FAK, respectively.

Statistical analysis

The data obtained from the image analyses were arranged and provided as the mean ± standard deviation. Statistical analysis was then performed with the software program IBM SPSS (IBM Corp., 2020. IBM SPSS Statistics for Windows: Version 27.0, NY, USA). A one-way analysis of variance (ANOVA) test was conducted to compare all groups and this was followed by Tukey's post hoc test when the ANOVA test revealed a significant difference. Significance was considered at p values < 0.05.

RESULTS

Double immunohistochemical expression of PCNA and FAK

All groups showed nuclear immunoreactivity for PCNA and cytoplasmic immunoreactivity for FAK **(Figure 1)**.

PCNA nuclear count immunoexpression

The CEOT group had the highest mean PCNA nuclear count, whereas the GOC group had the lowest, with a statistically significant difference between the groups ($P < 0.0001$). The CEOT group had a statistically significant higher mean nuclear count than the other groups. Additionally, the AB group had a higher mean nuclear count than unicystic AB and GOC with a statistical significance. Furthermore, in comparison to the GOC group, the unicystic AB group had a higher mean nuclear count ($P < 0.0001$; **Table 1**, **Figure 2**). Tukey post hoc of PCNA immunoexpression between each two groups revealed a significant statistical difference ($P<0.05$; **Table 2**).

FAK immunoexpression area percent

Among all the groups, the greatest mean area percent was recorded in the CEOT group, whereas the lowest value was observed in the unicystic AB group, with a statistically significant difference between the groups ($P < 0.001$). A statistically significant higher FAK immunoexpression in the CEOT group compared with the other groups. In addition, a statistically significant greater area percent was observed in the AB group compared to the unicystic AB and GOC groups. The mean area percent was significantly increased in the GOC group in comparison with the unicystic AB group ($P < 0.0001$; **Table 3**, **Figure 3**). Tukey post hoc of FAK immunoexpression between each two groups revealed a significant statistical difference ($P <0.05$; **Table 4**).

Comparing expressions of PCNA and FAK in double immunostained odontogenic lesions

The CEOT group displayed the greatest mean for both markers. However, the lowest means of immunoexpression for PCNA and FAK were in the GOC group and the unicystic AB group, respectively.

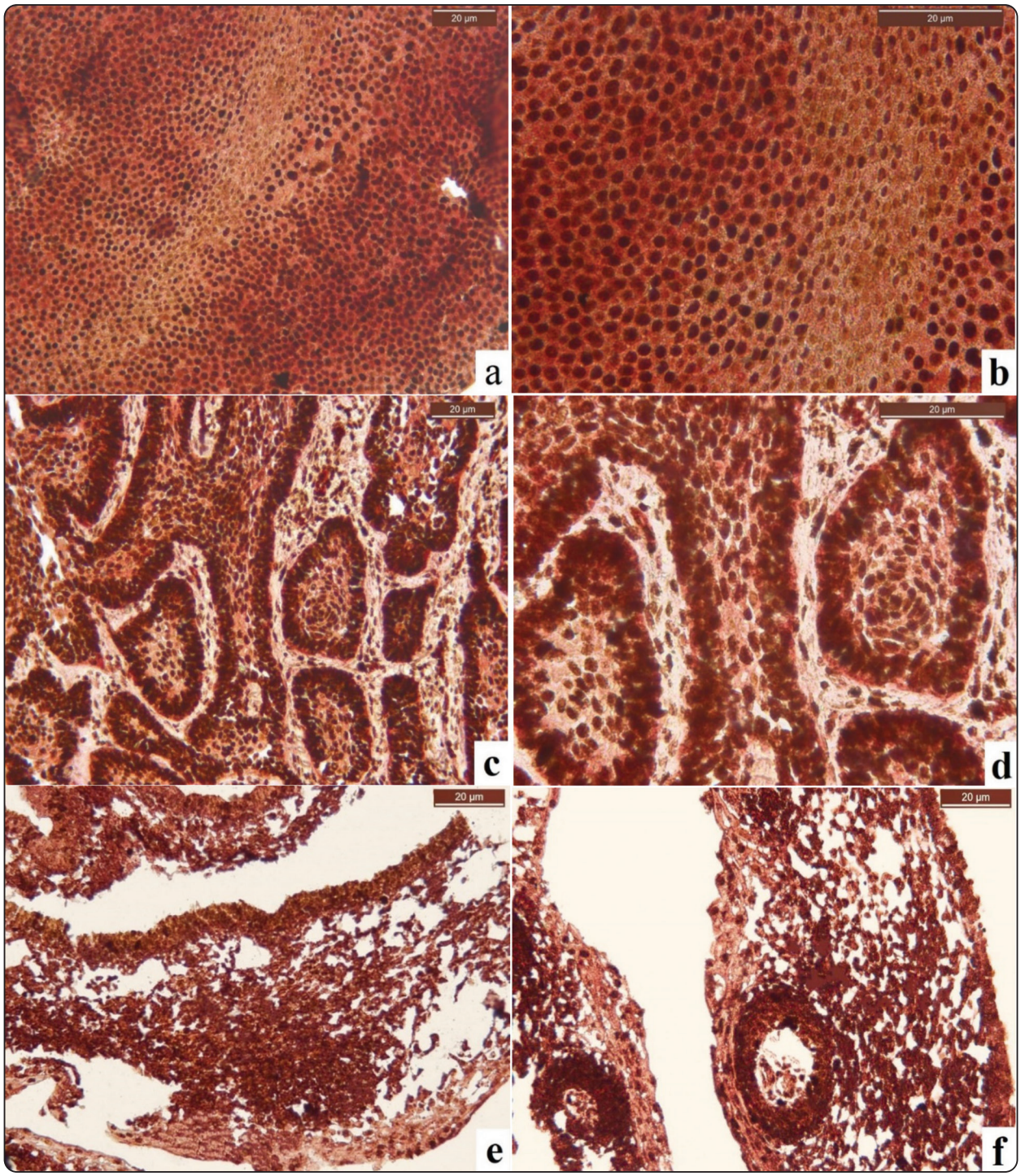

Fig. (1) Photomicrographs of anti-PCNA and anti-FAK double immunostained sections, showing positive nuclear PCNA expression and positive cytoplasmic FAK expression in odontogenic lesions: **(a)** calcifying epithelial odontogenic tumor (× 200). **(b)** a higher magnification of the same field (× 400). **(c)** follicular ameloblastoma (× 200). **(d)** a higher magnification of the same field (× 400). **(e)** unicystic ameloblastoma (× 200). **(f)** glandular odontogenic cyst immunostained in all cyst lining layers except for mucous cells (× 200).

TABLE (1) PCNA nuclear count in all groups and significance of the difference using ANOVA test.

P.O.C	AB	Unicystic AB	CEOT	GOC
Mean	146.4[a]	120.23[b]	465.6[c]	74[d]
SD	26.65	26.92	83.42	9.87
SE	4.95	4.99	19.37	1.83
Min	103	81	284	52
Max	194	187	653	90
F-value	450.563			
P-value	< 0.0001*			

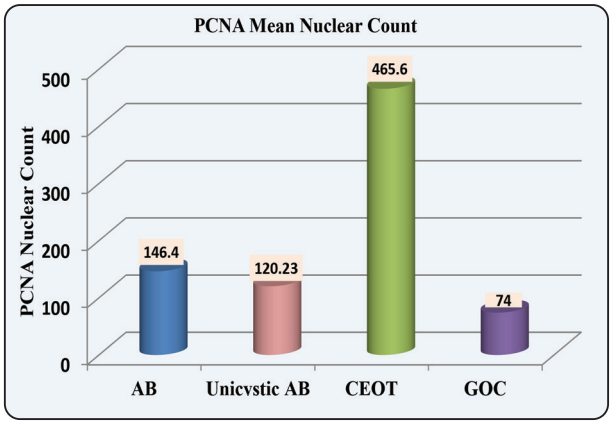

Fig. (2) Column chart showing the PCNA mean nuclear count of the conventional ameloblastoma (AB), unicystic AB, calcifying epithelial odontogenic tumors (CEOTs), and glandular odontogenic cysts (GOCs) groups.

TABLE (2) Tukey post hoc of PCNA immunoexpression between each two groups.

Difference between groups	Difference of means	SE of difference	95% CI	T-value	P-value
AB -Unicystic AB	26.1700	6.196	12.3262 to 40.0138	3.7840	0.0004
AB - CEOT	-319.2000	15.989	-351.2048 to -287.1952	19.9641	> 0.0001
AB - GOC	72.4000	5.189	62.0139 to 82.7861	13.9537	> 0.0001
Unicystic Ab- CEOT	345.3700	16.004	313.3351 to 377.4049	21.5806	> 0.0001
Unicystic AB- GOC	46.2300	5.235	35.7514 to 56.7086	8.8312	> 0.0001
CEOT - GOC	391.6000	15.337	360.9005 to 422.2995	25.5337	> 0.0001

Significant at p < 0.05.** **Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

TABLE (3) Area percent of FAK immunoexpression in all groups and significance of the difference using ANOVA test.

P.O.C	AB	Unicystic AB	CEOT	GOC
Mean	31.16[a]	14.80[b]	40.58[c]	17.44[d]
SD	5.68	3.36	5.88	4.75
SE	1.055	0.62	1.093	0.88
Min	21.54	9.54	28.43	9.33
Max	41.34	21.67	48.44	25.85
F-value	173.968			
P-value	< 0.0001*			

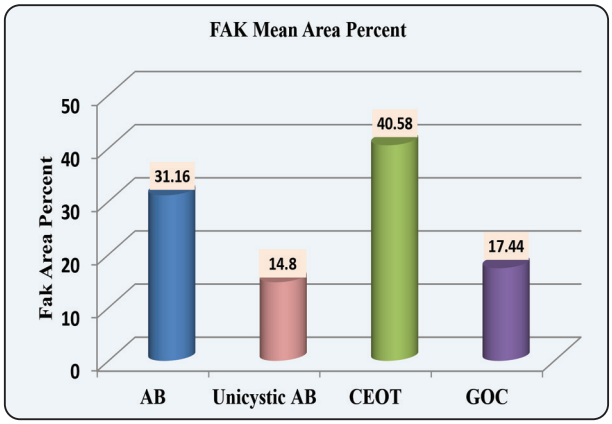

Fig. (3) Column chart showing the mean area percent of FAK immunoexpression in the conventional ameloblastoma (AB), unicystic AB, calcifying epithelial odontogenic tumors (CEOTs), and glandular odontogenic cysts (GOCs) groups.

TABLE (4) Tukey post hoc of FAK immunoexpression between each two groups.

Difference between groups	Difference of means	SE of difference	95% CI	T-value	P-value
AB -Unicystic AB	-16.3600	1.205	-18.7718 to -13.9482	13.5781	> 0.0001
AB - CEOT	9.4200	1.493	6.4322 to 12.4078	6.3111	> 0.0001
AB - GOC	-13.7200	1.352	-16.4260 to -11.0140	10.1491	> 0.0001
Unicystic Ab- CEOT	-25.7800	1.236	-28.2550 to -23.3050	20.8501	> 0.0001
Unicystic AB- GOC	-2.6400	1.062	-4.7664 to -0.5136	2.4853	0.0159
CEOT - GOC	-23.1400	1.380	-25.9025 to -20.3775	16.7674	> 0.0001

Significant at p < 0.05. Tukey's post hoc test: means sharing the same superscript letter are not significantly different.

DISCUSSION

Odontogenic benign neoplasms and cysts display diverse biological behaviors, extending from indolent encapsulated lesions to highly aggressive infiltrating and recurring lesions [3]. A CEOT, known as Pindborg's tumor, commonly shows high aggressiveness and rapid progression over a short duration despite its benign behavior [24]. A CEOT is similar to a conventional AB as they are benign epithelial odontogenic neoplasms with local invasiveness, although a conventional AB is the most aggressive odontogenic lesion with the highest tendency for recurrence [25]. Among the odontogenic cysts, GOC shows the highest aggressive behavior and recurrence rate and has great potential to reach large sizes [26].

It is worth mentioning that many preclinical and clinical trials involving PCNA and FAK antineoplastic therapy have been conducted[27–30], but none included odontogenic lesions. Therefore, the aim of this study was to evaluate the aggressiveness of select odontogenic lesions by analyzing the expression of PCNA and FAK with a double immunostaining technique and utilizing the evidence that they are highly expressed in aggressive and invasive lesions[31,32].

Interestingly, the comparative expression of both PCNA and FAK has not been done before for odontogenic lesions. Moreover, FAK was not previously studied in some odontogenic lesions. Furthermore, even though there is a general consensus regarding the aggressiveness of the selected lesions, up to date the comparative aggression of some of these lesions (such as GOC versus unicystic AB) remains debatable. Thereby, the current work demonstrates novelty utilizing a nice double immunostaining technique to examine the co-distribution of both PCNA and FAK (one cytoplasmic and one nuclear) in the selected odontogenic lesions. Among the studied odontogenic lesions in the current study, CEOT recorded the greatest immunoexpression profile of PCNA as well as FAK. Surprisingly, CEOT displayed higher expression levels of both markers, even more than AB mentioned in the literature as the most aggressive benign odontogenic lesion. No previous studies have evaluated double immunostaining of these markers for CEOT, and this might return to the highly aggressive nature of some CEOT lesions.

The conventional AB group showed the second-highest immunoexpression level of both PCNA and FAK compared with the CEOT group. Correspondingly, other studies have reported that AB had the highest expression of both markers among the studied odontogenic lesions and this was correlated to its aggressiveness [23,33,34]. In unicystic ABs, the results of the current study displayed a lower expression level for FAK than conventional ABs with a significant statistical difference. On the

contrary, Patil *et al*., as well as Bello *et al*., denoted similar strong expression patterns in conventional ABs and unicystic subtypes without statistically significant differences in FAK immunoexpression between them [23,33].

Regarding the expression of PCNA in the unicystic AB group, the current results revealed positive nuclear immunoreactivity in the cystic tumor lining, which displayed a lower mean nuclear count compared with the conventional AB group, with a significant statistical difference between the two groups. Accordingly, comparative studies assessed the percentage of PCNA immunostained cells in conventional ABs and unicystic subtypes and found that the lowest number of PCNA-positive nuclei was seen in unicystic ABs and strong expression in the conventional ABs with a significant statistical difference [34,35]. Thereby, the differences in PCNA and FAK expression levels between conventional ABs and unicystic ABs could imply differences in proliferation, aggressive natures, and recurrence rates among the two types of AB.

Due to the aggressive behavior of GOCs, and as the expressions of PCNA and FAK are suggestive for a high proliferative index, the current work examined the immunoexpressions of both markers in GOCs to ensure their possible role in the cyst aggressiveness. All the GOC cases showed positive nuclear expression for PCNA and positive cytoplasmic expression for FAK in cyst lining layers except for mucous cells, which in turn might exclude the role of such cells in the aggressiveness of cysts. Additionally, GOCs showed the least mean nuclear count of PCNA between all studied groups. In the available literature, no studies have examined the expression of FAK in GOCs. Meanwhile, Kaplan *et al*. evaluated PCNA expression in GOCs in comparison to low-grade mucoepidermoid carcinoma and found that there was no significant difference between them despite their differences in aggressive natures and recurrence rates [14].

CONCLUSIONS

In conclusion, positive immunohistochemical PCNA and FAK expressions were found in the selected odontogenic lesions with strong immunoexpression found in the CEOT and AB groups. Notably, this is the first study that employed PCNA and FAK double immunohistochemical staining to assess CEOTs. The results pointed to the possible role of PCNA and FAK double immunohistochemistry in aggressive odontogenic lesions, particularly CEOTs and ABs, considering both markers may be a clue to justify the proliferative activity and aggressiveness of these lesions.

Further studies should be conducted to correlate PCNA and FAK immunoexpression in odontogenic lesions, with particular concern for CEOTs and GOCs, which have been under-investigated. Hence, both markers may be useful in the future as routine examinations for assessment of aggressive lesions to assist with treatment planning and prognoses.

Limitations and recommendations

The scarcity of some odontogenic lesions such as CEOT and GOC posed some difficulty in enlarging the sample size. Therefore, the sample size was considered a limitation for this study, and to overcome this we recommend a multi-center study to ensure a larger sample size.

List of abbreviation

Ameloblastoma (AB)

Calcifying epithelial odontogenic tumor (CEOT)

Glandular odontogenic cyst (GOC)

Hematoxylin and eosin (H&E)

Proliferating cell nuclear antigen (PCNA)

Focal Adhesion Kinase (FAK)

One-way analysis of variance (ANOVA)

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