(RESEARCH ARTICLE)

Utilization of cell proliferation markers to diagnose cystic jaw pathologies

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Abstract

Markers of cell proliferation are widely used as diagnostic and prognostic tools. Coincident estimation of these markers increases the precise evaluation of the proliferative status of different tissues and can also be helpful in determining progression, aggressiveness and prognosis of the lesions. The current study investigated the expression of PCNA and MCM3 cell proliferation markers in 40 formalin fixed paraffin embedded tissue blocks of odontogenic keratocyst (OKC) and unicystic ameloblastoma (UA) cases using immunohistochemistry method. Markers` expression based on the intensity, percentage of positively stained cells and localization of reaction through the cyst lining epithelium was separately analyzed for each marker using Chi square test, the results of which were significant for the two markers (P < 0.05). Both markers revealed statistically significant differences between OKC and UA cases regarding markers expression intensity, positivity score and localization of reaction through the epithelium. Mural UA histologic variant was significantly different than luminal and intraluminal variants. The correlation coefficient between the two markers was found to be 0.86.

Keywords: Odontogenic keratocyst (OKC); Unicystic ameloblastoma (UA); PCNA; MCM3; Cell proliferation markers

1. Introduction

Odontogenic cysts and tumors are heterogeneous group of osteo-destructive lesions that had varied clinical and biological behavior [1]. OKCs are the most common developmental odontogenic cysts that originate from cell rests of dental lamina [2]. OKC has aggressive biological behavior and high recurrence rate that`s why the term keratocystic odontogenic tumor (KOT) was suggested by WHO classification in 2005 [3, 4]. However, WHO reclassified it again as OKC in 2017 because of insufficient evidence to support the neoplastic origin [5]. Microscopically, OKC is characterized by a palisaded basal cell layer of basophilic columnar cells and a surface of corrugated parakeratin. Presence of inflammation induces reactive changes in the cyst lining epithelium [6]. Previous studies have suggested that increased epithelial activity in OKC is responsible for the aggressiveness of this lesion in comparison with other odontogenic cysts [7, 8].

Among the epithelial odontogenic tumors, ameloblastoma is a common benign locally aggressive neoplasm that has a high rate of recurrence [9]. WHO histological classification of odontogenic tumors classified ameloblastoma into intraosseous solid or multicystic type, unicystic type, and peripheral types [10]. UA is generally biologically less aggressive and responds better to enucleation or curettage than other types of ameloblastoma [11]. Three histopathologic variants may be seen; luminal, intraluminal, and mural UAs [12]. Prognostic variation is recorded between these histologic variants that need various treatment modalities. While the luminal variant does not infiltrate into the surrounding bone, the mural variant sometimes shows deep invasion into the cyst wall and needs resection and long-term follow up [13]. UA may also present with squamous metaplasia, and sometimes by a nondescript epithelium, which can create diagnostic confusion with odontogenic cysts especially OKC [14]. Thus, the histologic presentation of

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UA can be mistaken for OKC [15]. Both are characterized by a structure similar to other non neoplastic cystic lesions and also share many clinical and radiological features [16]. Consequently, it is necessary to include them in differential diagnosis to understand the similarities and differences among them that could be useful for diagnosis and therapeutic purposes [6 - 9].

Cell proliferation plays a basic role in cell growth and maintenance of tissue homeostasis, and also in several biological and pathological events, such as tumor development [17, 18]. The use of tumor markers to differentiate between OKC and UA can be helpful for accurate diagnosis as their prognosis and treatment modality differs. Proliferating nuclear cell antigen (PCNA) is known as an important protein in DNA synthesis and repair [19, 20]. This nuclear non-histone protein is an accessory protein for DNA polymerase alpha, an essential factor for DNA replication and repair. This protein is elevated during the G1/S phase [21]. Currently, new markers are being added to evaluate cell proliferation. Minichromosome maintenance (MCM) proteins family consists of eight members. They form a ring-shape complex that is involved in the initiation and elongation of DNA replication. They also prevent replication and maintain the genome integrity [22, 23]. These proteins are expressed during all cell cycle phases in dividing cells, but are not detectable in quiescent cells (G0 phase) that`s why MCMs are used for evaluating tumor behavior [17]. To understand the differences between OKC and UA that could be useful for diagnosis and therapeutic purposes, the current study evaluated PCNA and MCM3 immunohistochemical expression in OKC and the three histologic variants of UA.

2. Material

The present study was applied on 40 paraffin embedded tissue blocks of OKCs (20 cases) and UAs (20 cases) that retrieved along with their medical records from archival files of Oral Pathology Department, Faculty of Dentistry, Mansoura University. All the included cases had complete medical records and confirmed diagnosis. Cases that had insufficient biopsy specimens and those with missing medical records were excluded from the study.

2.1. Methods

2.1.1. Clinical data retrieval

Patients` clinical data was gathered from their medical reports. The current research involved 25 males and 15 females with 1.66:1 male to female ratio. The age of 60% of the worked cases was less than 30 years. Four of the studied cases (10%) had tumors aroused from the maxilla, while the rest of cases (36 cases, 90%) were from the mandible. Mandible posterior molar ramus was the most common site of OKC and UA cases (33 cases, 82.5%). Only 3 cases aroused from mandible anterior and 4 cases aroused from maxilla. Eleven cases had pathologies smaller than 5 Cm in diameter. On the other hand, pathologies of large size (larger than 5 Cm in whole diameter) reported in 29 cases (72.5%). Twelve cases (60%) of OKC had multilocular radiographic presentation. On the other hand, 8 OKCs (40%) appeared as unilocular radiolucency that had similar presentation of UA cases. The greater number of OKCs (13 cases, 65%) and 9 cases of mural UA variant (90%) demonstrated diffuse ill-defined radiographic margin. Conversely, all UA cases of luminal and intraluminal histologic variant presented well defined radiographic margin. The studied patients` clinicopathological characteristics illustrated in (table 1).

2.1.2. Immunohistochemical staining

Two 4 microns thick sections were cut from each paraffin block for immunostaining to study the expression of PCNA and MCM3 in the selected cases. Sections were mounted on positive charged slides. Immunostaining was performed using Avidin Biotin Complex (ABC) method according to the manufacturer's instructions. The slides were deparaffinized then rehydrated in descending grades of alcohol and then water. Treatment of sections with 0.5% H_2O_2 in methanol (30 minutes) to block the endogenous peroxidase activity and then washed in phosphate buffer saline (PBS; 5 minutes). Pretreatment of the tissue sections by immersing in boiling citrate at 94 ˚c then cooled at room temperature then washed with distilled water. Followed by incubation of the slides in a solution of protease XIV 50 mg in 100 ml of 0.1 M, PH 7.4 PBS (pre warmed to 37˚c) for 15 minutes at 37 ˚c then washed 3 times in PBS for 5 minutes to digest the proteolytic enzyme activity. Blocking of non-specific binding of antibody by incubating the slides in 4.0 % mouse serum for 30 minutes at room temperature. The primary antibody was applied and the slides were incubated with the primary antibody for overnight at room temperature then sections were washed 3 times in PBS for 5 minutes. ABC complex peroxidase solution was applied according to manufacturer's directions. Sections were incubated for 30 minutes at room temperature then washed 3 times in PBS for 5 minutes. Application of chromogen for development of colored reaction product was done by using 3.3' diaminobenzidine-4HCL (DAB) 1mg/ml in PBS supplemented with H_2O_2 from Sigma chemical Co. St. Lo Missoure (10 µl of 50% H_2O_2 in 5 ml PBS). The DAB chromogen yielded a brown reaction end product at the site of target antigen. The sections were counter stained with Mayer's hematoxyline and were covered using Canada balsam. Positive controls of the used antibodies were performed by staining sections of breast

cancer at the same time and under the same conditions. Negative control slides obtained by replacement of the primary antibodies by plain PBS.

Table 1 Clinicopathological criteria of the studied OKC and UA cases

Clinicopathologic parameters	Variables	OKC (No. cases)	UA (No. cases)	Pearson Chi Square test (P)
UA variant	Luminal		7	
	Intraluminal		3	
	Mural		10	
Gender	Male	11	14	0.034
	Female	9	6	
Age groups	Less than 30 years	12	12	0.004
	More than 30 years	8	8	
Site	Mandible	19	17	0.106
	Maxilla	1	3	
Antroposterior jaw localization	Mandible posterior molar ramus	18	15	0.296
	Mandible anterior	1	2	
	Maxilla	1	3	
Pathology radiographic size	< 5 Cm	2	9	0.006
	≥ 5 Cm	18	11	
Unilocular/ Multilocular radiolucency	Unilocular radiolucency	8	20	0.001
	Multilocular radiolucency	12	0	
Radiographic margin	Diffuse ill-defined margin	13	9	0.000
	Well defined margin	7	11	
Inflammation	Present	8	16	0.018
	Absent	12	4	
Total (No. cases)		20	20	40

2.1.3. Evaluation and scoring of immunohistochemical reaction

Immunohistochemical assessment of PCNA and MCM3 expression was performed by scanning each slide under low magnification (×100) to identify regions containing positive immunoreactivity. At high magnification (×400), immunostaining was evaluated using a method described by Deraco et al (2015) [24]. Briefly, the results were independently evaluated based on staining intensity and the proportion of positively stained cells. The positive expression of PCNA and MCM3 was localized in the nucleus. Staining intensity score was categorized as negative (score 0), weak (score 1), moderate (score 2), and strong (score 3). The percentage of positive cells was independently scored based on the interquartile range as follows: Score (0) if the percentage of positive cells < 15%, Score (1) the percentage

of positive cells ≥ 15 and < 30%, Score (2) the percentage of positive cells ≥ 30% and < 50%, Score (3) the percentage of positive cells ≥ 50 and < 70%, Score (4) the percentage of positive cells ≥ 70%.

A final score based on adding two score values was classified as negative (score 0), weakly positive (score 1-2), positive (score 3-4), strongly positive (score 5-6). Those with a final score of < 3 were defined as 'low expression' and those with a final score of ≥ 3 were considered 'high expression'.

3. Results

3.1. Histopathological findings

The present study included 40 cases that categorized as 20 OKCs (50%) and 20 UAs (50%). OKC revealed a cyst wall formed of thin and even thickness lining epithelium with corrugated parakeratin surface. Its basal cell layer composed of hyperchromatic tall columnar cells with reversed polarity. The connective tissue wall comprised of parallel arranged collagenous fibers with few cells and blood vessels. UAs presented as a single cystic cavity lined by ameloblastomatous epithelium. Histological examination of the UA variants revealed that mural type was the predominant histological variant. One half of the studied UA cases (10 cases out of 20) were mural variant. Mural UA characterized by cystic cavity lined with odontogenic epithelium in addition to definite ameloblastoma tumor islands within the fibrous connective tissue wall. Seven cases of UA (35%) were diagnosed as luminal histological variant where the lining epithelium exhibited columnar differentiation and reverse polarization of the basal cell layer. Intraluminal variant represented by 3 cases (15% of UA). It consisted of odontogenic epithelium lining and fibrous connective tissue wall, with tumor extending into the cystic luminal space (Fig. 1).
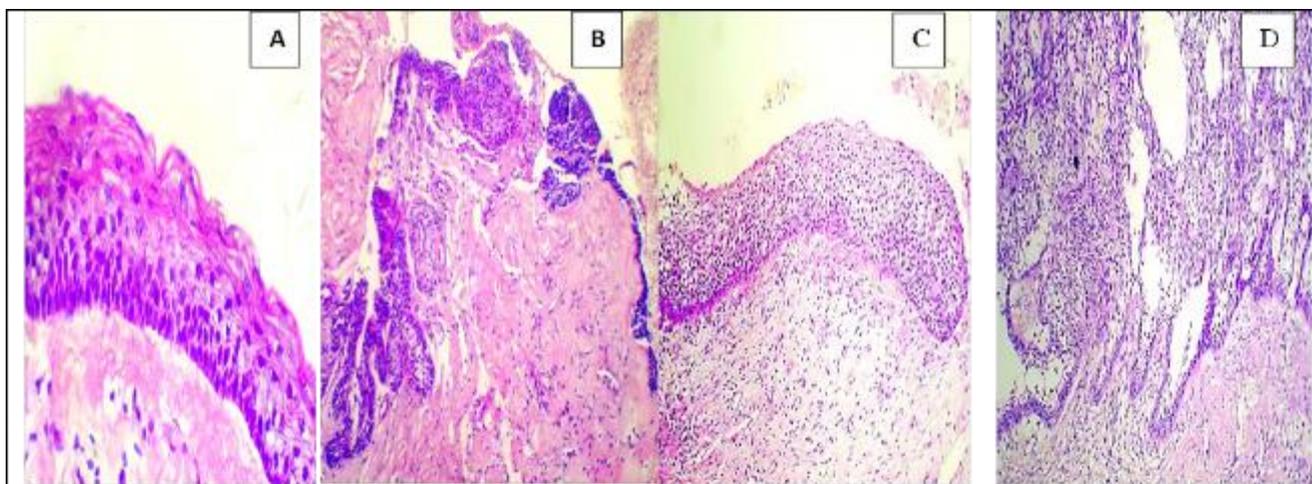

Figure 1 Photomicrographs show H&E stained sections of (A) OKC, (B) mural UA, (C) luminal UA and (D) intraluminal UA variant (H&E x 250)

3.2. Immunohistochemical findings

3.2.1. Expression of PCNA and MCM3 in OKC and UA

PCNA and MCM3 immunohistochemical expression observed as brown staining in the nuclei of cells. PCNA and MCM3 expression in OKC cases was confined mainly in suprabasal layers and basal cell layer of the cyst lining epithelium (Fig. 2). In UA cases, both markers showed similar pattern of expression. Mural UA variant revealed expression through the full thickness of epithelium lining the cyst and ameloblastoma follicles (Fig. 3). Luminal and intraluminal UA variants showed immunoreactivity through the full thickness of epithelium (Fig. 4).
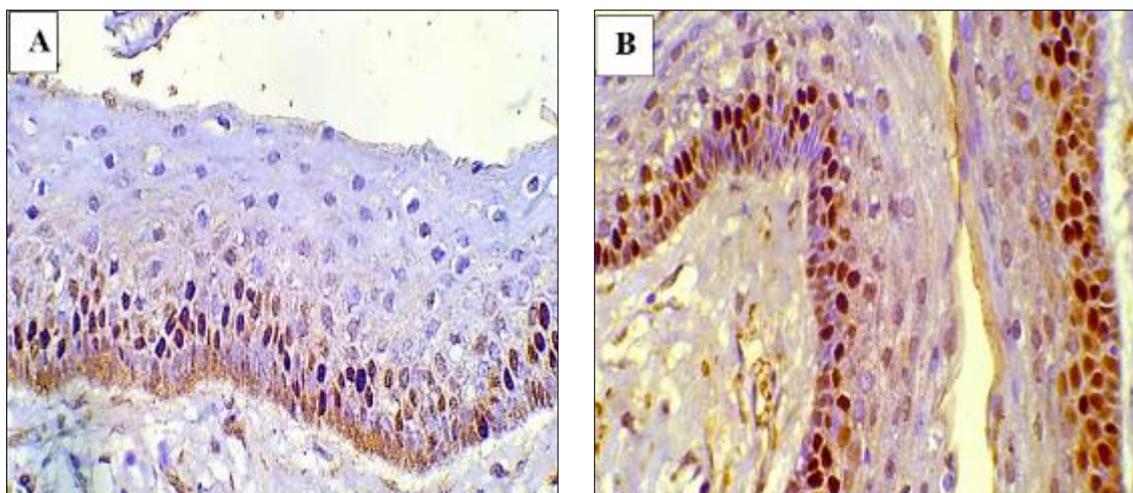
Figure 2 Strong basal and suprabasal expression of PCNA (A) and MCM3 (B) in OKC (ABC- DAB x400)

3.2.2. Comparison between expression of PCNA in OKC and UA

A) PCNA positivity of expression score

About two thirds of OKCs (13 cases, 65%) demonstrated (score 4) positivity, while the rest of OKC cases showed (score 3) positivity. On contrary, 15 of UAs (75%) had (score 3) positivity, while the remaining 5 cases of mural histologic variant showed (score 4) positivity. Pearson Chi square test revealed that PCNA positivity of expression score was significantly different between OKC and UA cases (p= 0.009, table 2). Moreover, all the examined cases of luminal and intraluminal UA histologic variant had (score 3) positivity, while one half of mural UA cases had (score 3) and the remaining half had (score 4) positivity. Pearson Chi square test revealed a statistically significant difference among the three histologic variants of UA regarding PCNA positivity of expression score (p=0.036, table 3).
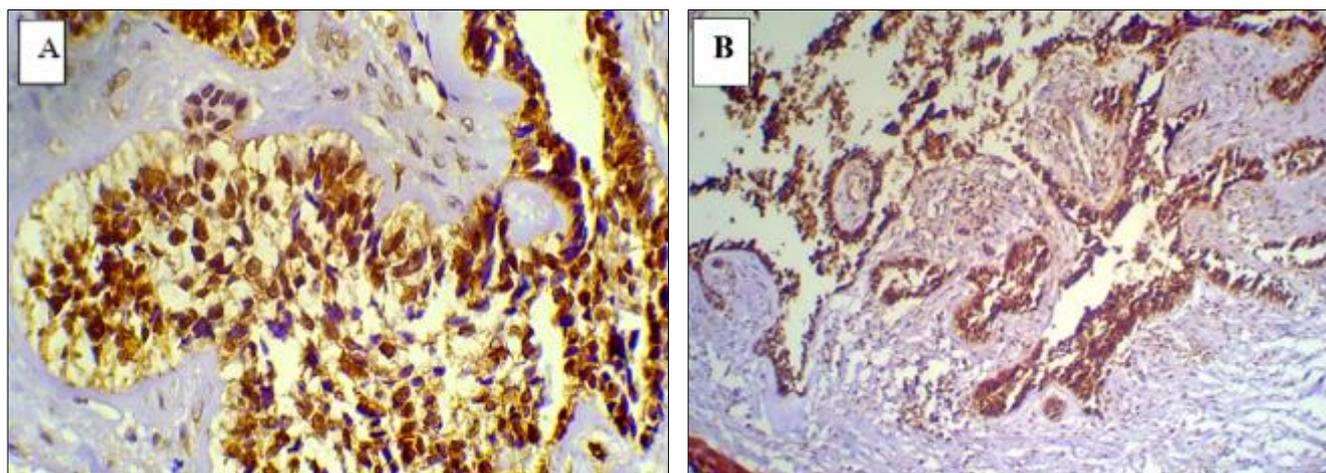

Figure 3 Mural UA variant shows strong intensity and highest positivity score (≥ 70%) of (A) PCNA and (B) MCM3 (ABC- DAB, x400, x250)

Table 2 PCNA positivity score in OKC and UA cases

| PCNA positivity score		Pathology histologic type				Total	Pearson Chi-Square
		Luminal UA	Lntraluminal UA	Mural UA	OKC		
> 50% and < 70%	Count	7	3	5	7	22	0.009
	% within pathology histologic type	100.0%	100.0%	50.0%	35.0%	55.0%	
	% of Total	17.5%	7.5%	12.5%	17.5%	55.0%	
> 70%	Count	0	0	5	13	18	
	% within pathology histologic type	0.0%	0.0%	50.0%	65.0%	45.0%	
	% of Total	0.0%	0.0%	12.5%	32.5%	45.0%	
Total	Count	7	3	10	20	40	
	% within pathology histologic type	100.0%	100.0%	100.0%	100.0%	100.0%	
	% of Total	17.5%	7.5%	25.0%	50.0%	100.0%	
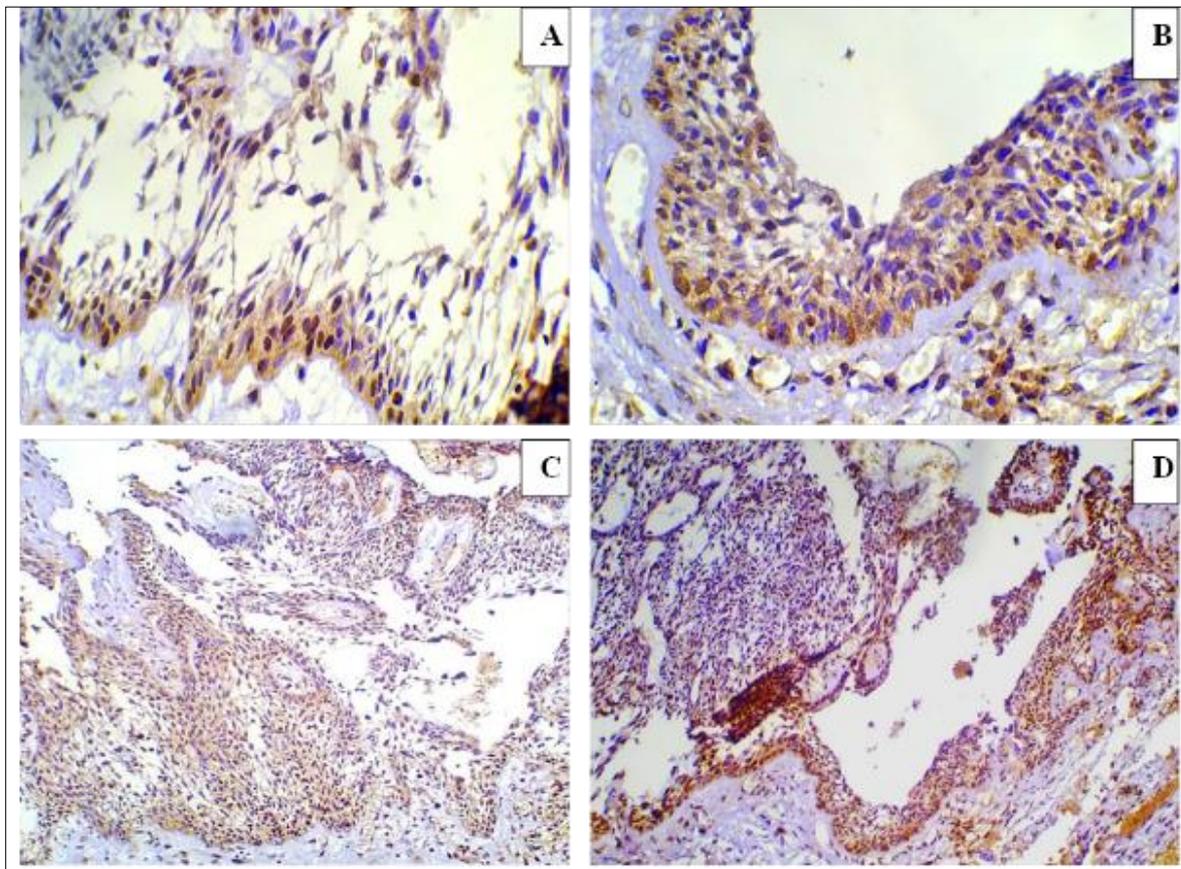
Figure 4 Moderate expression intensity and score (3) positivity of PCNA and MCM3 respectively in (A& B) luminal UA variant and (C&D) intraluminal UA variant (ABC- DAB, x400, x250)

Table 3 PCNA positivity score in UA variants

PCNA positivity score		Unicystic Ameloblastoma variants			Total	Pearson Chi-Square
		Luminal	Intraluminal	Mural		
> 50% and < 70%	Count	7	3	5	15	0.036
	% within unicystic ameloblastoma variants	100.0%	100.0%	50.0%	75.0%	
	% of Total	35.0%	15.0%	25.0%	75.0%	
> 70%	Count	0	0	5	5	
	% within unicystic ameloblastoma variants	0.0%	0.0%	50.0%	25.0%	
	% of Total	0.0%	0.0%	25.0%	25.0%	
Total	Count	7	3	10	20	
	% within unicystic ameloblastoma variants	100.0%	100.0%	100.0%	100.0%	
	% of Total	35.0%	15.0%	50.0%	100.0%	

B) PCNA expression intensity score

The majority of OKCs (17 cases, 85%) demonstrated strong PCNA expression intensity, while the rest of cases (3 cases, 15%) showed moderate expression intensity. Conversely, 15 UAs (75%) demonstrated moderate PCNA expression intensity, while the remaining 5 cases (25%) of mural UA variant presented strong expression intensity.

Table 4 PCNA intensity score in OKC and UA variants

PCNA expression intensity score		pathology histologic type				Total	Pearson Chi-Square
		luminal UA	Intraluminal UA	Mural UA	OKC		
Moderate	Count	7	3	5	3	18	0.000
	% within pathology histologic type	100.0%	100.0%	50.0%	15.0%	45.0%	
	% of Total	17.5%	7.5%	12.5%	7.5%	45.0%	
strong	Count	0	0	5	17	22	
	% within pathology histologic type	0.0%	0.0%	50.0%	85.0%	55.0%	
	% of Total	0.0%	0.0%	12.5%	42.5%	55.0%	
Total	Count	7	3	10	20	40	
	% within pathology histologic type	100.0%	100.0%	100.0%	100.0%	100.0%	
	% of Total	17.5%	7.5%	25.0%	50.0%	100.0%	

Pearson Chi square test revealed that PCNA intensity of expression was significantly different between OKC and UA cases (p= 0.000, table 4). Moreover, all the examined cases of luminal and intraluminal UA histologic variant presented moderate PCNA expression intensity, while one half of mural UA cases had strong intensity and the remaining half had moderate expression intensity. Pearson Chi square test revealed a statistically significant difference among the three histologic variants of UA regarding PCNA expression intensity (p=0.036, table 5).

Table 5 PCNA expression intensity score in UA variants

PCNA intensity score		Unicystic Ameloblastoma variants				Pearson Chi-Square
		Luminal	**Intraluminal**	**Mural**	**Total**	
Moderate	Count	7	3	5	15	0.036
	% within unicystic ameloblastoma variants	100.0%	100.0%	50.0%	75.0%	
	% of Total	35.0%	15.0%	25.0%	75.0%	
Strong	Count	0	0	5	5	
	% within unicystic ameloblastoma variants	0.0%	0.0%	50.0%	25.0%	
	% of Total	0.0%	0.0%	25.0%	25.0%	
Total	Count	7	3	10	20	
	% within unicystic ameloblastoma variants	100.0%	100.0%	100.0%	100.0%	
	% of Total	35.0%	15.0%	50.0%	100.0%	

3.2.3. Comparison between MCM3 expression in OKC and UA

A) MCM3 positivity of expression score

Table 6 MCM3 positivity score in OKC and UA

MCM3 positivity score		pathology			Asymp. Sig. (2-sided)
		UA	**OKC**	**Total**	
> 50% and < 70%	Count	14	5	19	0.004
	% within MCM3 positivity score	73.7%	26.3%	100.0%	
	% within pathology	70.0%	25.0%	47.5%	
	% of Total	35.0%	12.5%	47.5%	
> 70%	Count	6	15	21	
	% within MCM3 positivity score	28.6%	71.4%	100.0%	
	% within pathology	30.0%	75.0%	52.5%	
	% of Total	15.0%	37.5%	52.5%	
Total	Count	20	20	40	
	% within MCM3 positivity score	50.0%	50.0%	100.0%	
	% within pathology	100.0%	100.0%	100.0%	
	% of Total	50.0%	50.0%	100.0%	

The predominance of OKCs (15 cases, 75%) demonstrated (score 4) positivity, while the rest of OKCs showed (score 3) positivity. On contrary, 14 of UAs (70%) had (score 3) positivity, while the remaining 6 cases of mural histologic variant showed (score 4) positivity. Pearson Chi square test revealed that MCM3 positivity of expression score was significantly different in OKC than UA cases (p= 0.004, table 6). Moreover, all the examined cases of luminal and intraluminal UA histologic variant had (score 3) positivity, while 6 cases (60%) of mural UA had (score 4) and the remaining cases presented (score 3) positivity. Pearson Chi square test revealed a statistically significant difference among the three histologic variants of UA regarding MCM3 positivity of expression score (p=0.036, table 7).

Table 7 MCM3 positivity in UA variants

MCM3 positivity score		Unicystic Ameloblastoma variants				Pearson Chi square test
		Luminal	Intraluminal	Mural	Total	
> 50% and < 70% (score 3)	Count	7	3	4	14	0.014
	% within unicystic ameloblastoma variants	100.0%	100.0%	40.0%	70.0%	
	% of Total	35.0%	15.0%	20.0%	70.0%	
> 70% (score 4)	Count	0	0	6	6	
	% within unicystic ameloblastoma variants	0.0%	0.0%	60.0%	30.0%	
	% of Total	0.0%	0.0%	30.0%	30.0%	
Total	Count	7	3	10	20	
	% within unicystic ameloblastoma variants	100.0%	100.0%	100.0%	100.0%	
	% of Total	35.0%	15.0%	50.0%	100.0%	

B) MCM3 expression intensity score

Table 8 MCM3 expression intensity in UA and OKC

MCM3 expression intensity		pathology histologic type					Asymp. Sig. (2-sided)
		Luminal UA	Intraluminal UA	Mural UA	OKC	Total	
Moderate	Count	7	3	6	0	16	0.000
	% within MCM3 expression intensity	43.8%	18.8%	37.5%	0.0%	100.0%	
	% within pathology histologic type	100.0%	100.0%	60.0%	0.0%	40.0%	
	% of Total	17.5%	7.5%	15.0%	0.0%	40.0%	
Strong	Count	0	0	4	20	24	
	% within MCM3 expression intensity	0.0%	0.0%	16.7%	83.3%	100.0%	

	% within pathology histologic type	0.0%	0.0%	40.0%	100.0%	60.0%	
	% of Total	0.0%	0.0%	10.0%	50.0%	60.0%	
Total	Count	7	3	10	20	40	
	% within MCM3 expression intensity	17.5%	7.5%	25.0%	50.0%	100.0%	
	% within pathology histologic type	100.0%	100.0%	100.0%	100.0%	100.0%	
	% of Total	17.5%	7.5%	25.0%	50.0%	100.0%	

All the examined OKCs demonstrated strong MCM3 expression intensity. Conversely, 16 UAs (70%) demonstrated moderate MCM3 expression intensity, while the remaining 4 cases (30%) of mural UA variant presented strong expression intensity. Pearson Chi square test revealed that MCM3 intensity of expression was significantly different between OKC and UA cases (p= 0.000, table 8). Moreover, all the examined cases of luminal and intraluminal UA histologic variant presented moderate MCM3 expression intensity. Six cases of mural UA showed moderate expression intensity 4 cases had strong expression intensity. Pearson Chi square test revealed a statistically significant difference among the three histologic variants of UA regarding MCM3 expression intensity (p=0.036, table 9).

Table 9 MCM3 expression intensity in UA variants

MCM3 expression intensity		Unicystic Ameloblastoma variants				Pearson Chi square test (P)
		Luminal	Intraluminal	Mural	Total	
Moderate	Count	7	3	6	16	0.082
	% within unicystic ameloblastoma variants	100.0%	100.0%	60.0%	80.0%	
	% of Total	35.0%	15.0%	30.0%	80.0%	
Strong	Count	0	0	4	4	
	% within unicystic ameloblastoma variants	0.0%	0.0%	40.0%	20.0%	
	% of Total	0.0%	0.0%	20.0%	20.0%	
Total	Count	7	3	10	20	
	% within unicystic ameloblastoma variants	100.0%	100.0%	100.0%	100.0%	
	% of Total	35.0%	15.0%	50.0%	100.0%	

3.2.4. Correlation between PCNA and MCM3 expression in the studied cases

Relatively, the immunohistochemical expression of both markers demonstrated similar findings in the studied cases of OKC and UA. Spearman correlation coefficient test presented a strong positive correlation between PCNA and MCM3 expressions in the studied cases. Spearman's rho correlation coefficient was found to be 0.86 (table 10).

Table 10 Correlation between PCNA and MCM3 expression in the worked cases

			PCNA	MCM3
Spearman's rho	PCNA expression score	Correlation Coefficient	1.000	0.860**
		Sig. (1-tailed)	.	0.000
		N	40	40
	MCM3 expression score	Correlation Coefficient	0.860**	1.000
		Sig. (1-tailed)	0.000	.
		N	40	40

**. Correlation is significant at the 0.01 level (1-tailed).

4. Discussion

Odontogenic keratocyst (OKC) is the most common developmental odontogenic cyst that has a unique locally aggressive behavior and high recurrence rate. Short time ago, World Health Organization (WHO) classified it as a cystic odontogenic tumor (Keratocystic odontogenic tumor) [25]. Increased epithelial activity in OKC is responsible for the aggressiveness of this cyst in comparison with other odontogenic cysts [7]. Ameloblastoma is the most common odontogenic tumor. It also has a locally invasive behavior and increased tendency to recur after removal [7]. Unicystic ameloblastoma is a superficially invasive form of ameloblastoma that characterized biologically to be less aggressive and has better response to enucleation and curettage than conventional ameloblastoma [26, 11]. Histologically, UAs are lined by a variable epithelium ranging from one that has typical ameloblastic characteristics to one that is metaplastic which appears completely nondescript consisting of several layers of nonkeratinizing squamous cells. In fact, such squamous metaplasia is frequent phenomenon in unicystic ameloblastomas and many of these lesions are lined predominantly by such nondescript epithelium [27]. In such cases, the differentiation between odontogenic cysts and UAs can be problematic as UAs could be mistaken for OKCs especially due to the overlapping of clinical and radiographic presentation of both lesions [14, 6].

Cell proliferation plays vital role in cell growth and maintenance, and also in several biological and pathological events, such as tumor development and tissue haemostasis [17, 18]. Identification of cell proliferation markers could be a useful diagnostic and prognostic tool to understand and predict the clinical and biological behavior of many pathologic lesions [28]. Proliferating nuclear cell antigen (PCNA) is an important protein in DNA synthesis and repair [19, 20]. There are numerous studies still using PCNA as the first choice marker of cell proliferation. Many investigations of tumor-cell proliferative activity have used PCNA and Ki-67 to evaluate cell proliferation in oral tumors [29- 31]. Currently, new markers are being added to evaluate cell proliferation. Minichromosome maintenance (MCM) proteins family consists of eight members. These proteins are expressed during all cell cycle phases in dividing cells, but are not detectable in quiescent cells (G0 phase) that`s why MCMs are used for evaluating tumor behavior [32]. Expression of MCM3 as a member of MCM2-7 complex has been established in several human neoplasms, but few studies were applied on lesions of odontogenic origin [33, 34]. The present study is a trial to through a beam of light to compare between OKC and UA of different histological variants to clarify any correlation or distinction between both lesions in order to detect the differences that could be a tool for their differential diagnosis. Simultaneous evaluation of PCNA and MCM3 can be a precise estimation for the proliferative activity of cells that can also be helpful in determining progression, aggressiveness and prognosis of the lesions.

The results of the current study showed statistically significant differences between OKC and UA in PCNA and MCM3 expression at the levels of percentage of positivity, expression intensity and localization of reaction through the epithelium ($p < 0.05$). Moreover, there were statistically significant differences in markers expression among the three histologic variants of UA; mural UA variant demonstrated different expression when compared to luminal and intraluminal variants. Relatively, both antibodies had the same level of expression. Spearman`s correlation coefficient test revealed a strong positive correlation between PCNA and MCM3 immunohistochemical expression in the studied OKC and UA cases (Spearman Rho`s correlation coefficient = 0.86). PCNA and MCM3 positivity of expression score could be used not only to differentiate between OKC and UA cases, but also between the three histologic variants of UA. Predominantly, OKCs showed the highest expression positivity score (> 70% positively stained cells). On the other hand, the greater number of UA cases had positivity score (>50% and ≤70%). Pearson chi square test revealed a statistically significant difference between OKCs and UAs according to PCNA and MCM3 positivity score. Moreover, mural UA histological variant showed the highest PCNA and MCM3 positivity score 4 (> 70% positively stained cells; 6 cases, 60%

of mural UA cases), while the other histological variants revealed only score 3 (>50% and ≤70%) positivity (100% of luminal and intraluminal histological variants). Chi square test revealed statistically significant differences among the studied UA histological variants regarding PCNA and MCM3 positivity of expression score (P< 0.05).

In agreement with our finding, Jaafari Ashkavandi et al., found that all specimens of dentigerous cyst (DCs), OKCs and ameloblastoma were positive for MCM3 and the expression of MCM3 were significantly higher in OKCs and ameloblastoma than in DCs. Also there was higher expression of MCM3 in OKCs than UAs [35]. These results indicated higher proliferative activity of ameloblastoma and OKCs. As these two entities had high recurrence rate and clinically aggressive behavior. Moreover, our findings support the findings of other researchers. Guler et al., and Nadalin et al., had reported lower proliferation activity of DC in comparison with OKC using Ki-67 and MCM2 markers [18, 36]. Nafarzadeh et al., found lower expression of Ki-67 and PCNA in DC compared to ameloblastoma [37]. Thosaporn et al., evaluated proliferation activity in DC, OKC and orthokeratinized odontogenic cyst (OOC) using IPO-38 and demonstrated a significant difference between DC, OKC and OOC [32]. Furthermore, on the same line of our findings COŞARCĂ et al., reported higher positive values in OKC than in DC using MCM3 proliferative marker. Although both were categorized as benign cystic lesions, but OKC had more aggressive behavior [38, 39]. Lameira et al., examined MCM3 and Ki67 expression in all AMs. They reported high MCM3 expression in all AMs and concluded that MCM3 could be a better marker of proliferation than Ki-67 [40]. Based on these results, cell proliferation markers as MCM3, PCNA, ki67 could be helpful for monitoring early stages of cell cycle to identify cells that are in G0 phase with the potential to enter the cell cycle and to identify proliferating cells [41]. Expression of PCNA and MCM3 could be more reliable marker of proliferation in assessing tumor growth and evaluating the potential for recurrence [42].

High positivity and intensity of expression of the studied proliferation markers was noted in OKCs and Mural UA variant. This mean that the expression of the used markers was linked to increased migration, invasion, and proliferation. Our results can support the conclusion that PCNA and MCM3 are better markers to evaluate tumoral behavior and more sensitive markers for the identification of proliferating cells. In harmony with our finding, Lau et al. [43] found that low MCM2, MCM3, and MCM7 expression levels in medulloblastoma modified cells (cultured in agar) correlated with decreases in the invasion and migration of these cells. MCM2, MCM3, and MCM7 overexpression have been linked to increase migration, invasion, and proliferation. Carreón-Burciaga RG et al also found that Ki-67, MCM2 and MCM3 expression levels were higher in AC than in UA and SMA, which indicate aggressive, invasive, and metastatic neoplasm [17].

Regarding PCNA and MCM3 expression intensity, the majority of OKCs revealed strong PCNA and MCM3 expression intensity. Moreover, mural variant was the only UA histological variant that demonstrated strong expression intensity. Pearson Chi square test revealed a high statistically significant difference between OKC and UA regarding PCNA and MCM3 expressions intensities. Furthermore, there were statistically significant differences among the studied UA histological variants regarding PCNA and MCM3 expression intensities using Chi square test (P< 0.05). Intensely stained cells localized in basal and parabasal cell layer in epithelium lining OKCs and peripheral ameloblast like cells in UA cases. These cells demonstrated high expression intensity as they are metabolically active (Increased PI) similar to ameloblast of enamel organ that had significant high expression of Ki-67 than the central cells [44, 45].

Gonzalez et al., [46] also demonstrated MCM 2, MCM 3 and Ki 67 mainly in the peripheral layer of the epithelial islands and cysts and a few positive in the more central areas [46]. This suggests that the growth of AM is produced by the peripheral expansion of the follicles and that this pattern of proliferation possibly indicates some degree of central maturational activity [44].

Immunohistochemical expression of PCNA and MCM3 revealed nuclear brown staining in the worked cases of OKC and UAs. Jaafari Ashkavandi et. al., Shahela et al., and Thosaporn et al., showed similar finding [35, 45, 32]. MCM3 nuclear localization was explained by Hong Yan et al, as follow, MCM3 proteins are temporally regulated with respect to the cell cycle. These proteins enter the nucleus at the end of mitosis, persist there throughout G phase, and disappear from it at the beginning of S phase. Once inside the nucleus, a fraction of MCM3 proteins becomes tightly associated with DNA. The association of MCM3 with chromatin presumably leads to the initiation of DNA synthesis, and their subsequent disappearance from the nucleus presumably prevents re-initiation of DNA synthesis at replication origins. This temporally and spatially restricted localization of MCM3 in the nucleus may serve to ensure that DNA replication occurs once per cell cycle [47]. PCNA is known as an important protein in DNA synthesis and repair [19, 20]. This nuclear non-histone protein is an accessory protein for DNA polymerase alpha, an essential factor for DNA replication and repair. This protein is elevated during the G1/S phase [21].

Another notable finding in our study is the localization of PCNA and MCM3 through the epithelium. Relatively, both proliferation markers demonstrated the same localization of expression through the epithelium in the worked OKC and

UA cases. OKCs showed basal and parabasal expression, while UA variants revealed expression through the full thickness of epithelium lining the cyst. Pearson chi square test revealed a statistically significant difference between OKCs and UAs according to localization of expression. Consistent to our finding, Zohreh Jaafari Ashkavandi et al., and Coşarcă et al., reported that MCM3 was expressed in basal and parabasal layers of the DCs and OKCs [35, 38]. Some other studies also demonstrated higher expression of proliferation markers in suprabasal layer of OKC in comparison with peripheral cells of ameloblastoma [32, 48]. Although both lesions are benign, but OKC is biologically more aggressive as the development of this type of cyst being based on maintenance of the proliferative capacity of cells in the parabasal layer [37, 39]. Aladim Gomes Lameira and his colleagues, concluded findings support ours. They found that proliferation marker (MCM3) expression was confined to basal and parabasal layers in normal mucosa and explained their finding as basal and parabasal cells characterized by high division capability than the mature fully differentiated cells [40].

On the same line to our findings in UA cases, PCNA and MCM3 expression was observed in full thickness of epithelium and in ameloblastoma follicles, Carreón-Burciaga et al., reported that MCM3 may present in non proliferating cells, but its signals were in a readiness to enter the cell cycle [17]. Furthermore, Zohreh Jaafari Ashkavandi and his team work reported that MCM3 expression in ameloblastomas was mostly found in peripheral ameloblast like cells and also in stellate reticulum (SR) like cells (with lower rate) [35]. These results were consistent with Endl et al. study, which suggested that MCM3 expression was capable to distinguish cells with the capacity to reenter cell division and to detect proliferating cells early [41]. They also reported that high expression level was predominated in areas of higher cellular density and in peripheral cells with columnar morphology, while in the central polyhedral cells, expression was minimal or absent [17]. This pattern of expression is owing to; the more differentiated the cells, the less proliferative activity is seen, so the expression of PCNA and MCM3 proteins are predominated in basal and parabasal cell layers where the cells characterized by high division capability, while the overlying layers of epithelium revealed lower level of cell division as the cells undergoing maturation and differentiation. For this reason, in normal epithelium, the expression is limited to the basal and parabasal cell layers; however, in dysplastic samples more cells are in the cell cycle. Due to loss of cell cycle control mechanisms in malignant cases, a greater number of cells and in more upper layers of the epithelium were positive for proliferation markers as MCM3 [40].

Although enzymatic activity of stromal components is a cause of aggressiveness in OKC and ameloblastoma, it has been maintained that the aggressive behavior of these tumors is also due to the epithelial characteristics [7]. In this view, some researchers have reported that up-regulation of urokinase plasminogen activator (UPA) and mutation of RAS protein in ameloblastoma, have resulted in the activation of mitogen-activated protein kinase (MAPK) pathway, increasing proliferation activity [49, 50]. Also, one study have showed that in association with increased proliferation rate (high Ki-67 index) in the epithelium of OKC, substance p (SP) and its receptor (NK-1R) are overexpressed [51]. Activation of NK-1R by SP can simulate mitogenic signals and induce proliferation [44]. Antagonists of NK-1R have also showed the inhibition of angiogenesis and cell migration and therefore, can be used for anticancer treatments [52].

5. Conclusion

In the process of cell proliferation, there is a need for cell division under the control of molecules as PCNA and MCM3 that expressed during cell cycle. Imbalance or increase of cell proliferation have been reported in various lesions such as tumors and cysts. It is also expected that lesions with high levels of invasion, the amount of molecules involved in cell cycle be different to those with less invasion. The present study evaluated PCNA and MCM3 immunohistochemical expression to discriminate between confusing cases of OKC and UA as both lesions are benign cysts that in some situations have an overlapping clinical, radiographic and histological feature. Many UAs could be mistaken for OKCs. Prognostic variation is recorded between these two entities and also among different histologic variants of UA that need various treatment modalities. Our results support the conclusion that PCNA and MCM3 are the best markers to evaluate tumoral behavior and more sensitive marker for identification of proliferating cells. We recommend using PCNA and MCM3 immunohistochemical expression to discriminate confusing cases of OKC and UAs to reach definitive diagnosis.

Compliance with ethical standards

Disclosure of conflict of interest

Authors have declared that no competing interests exist.

Statement of ethical approval

As per international standard or university standard, patient's written consent has been collected and preserved by the authors. Our study was approved by the faculty ethics committee.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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