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Double Immunohistochemical Expression of FAK and PCNA in Odontogenic Keratocyst, Orthokeratinized Odontogenic Cyst, Dentigerous Cyst and Glandular Odontogenic Cyst

Short running title: FAK and PCNA Double Immunostaining in Odontogenic cysts.

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Abstract:

Background: Odontogenic cysts are distinctive disorders that affect oral and maxillofacial tissues.The majority of odontogenic cysts such as dentigerous cysts and orthokeratinized odontogenic cyst show an indolent course, whilst others demonstrate an aggressive behavior and may have higher recurrence rate like odontogenic keratocysts and glandular odontogenic cysts.Here wecompared FAK and PCNA expression in the previously mentioned cysts and attempted to correlate the findings to their variable clinical behaviors.

Aim of the study:This study aimed to clarify the role of epithelial to mesenchymal transition in invasion potential, proliferation and aggressiveness of these lesions, which had been achieved.

Methods: Double Immunostaining technique for FAK and PCNA was performed on the same tissue specimens using two types of chromogens. DAB chromogen was used for the assessment of nuclear expression of PCNA, while AEC chromogen was used for the assessment of cytoplasmic expression of FAK.

Results: The greatest mean of the nuclear immunoexpression of PCNA was recorded in glandular odontogenic cyst group, followed by the odontogenic keratocyst, while the greatest mean area percent of the cytoplasmic expression of FAK was recorded in odontogenic keratocyst group, followed by the glandular odontogeniccyst.

Conclusions:Odontogenic keratocyst has the highest epithelial to mesenchymal transition ability followed by glandular odontogenic cyst, while glandular odontogenic cyst has the highest proliferation activity followed by odontogenic keratocyst. On the other hand, orthokeratinized odontogenic cyst and dentigerous cyst have the lowest epithelial to mesenchymal transition ability and proliferation activity, which can explain the diverse behavior of those odontogenic cysts.

Keywords: Odontogenic cysts; Double immunostaining; FAK; PCNA.

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Introduction:

Odontogenic cysts encompass up to 90% of jaw cysts. The majority of odontogenic cysts such as dentigerous cysts (DCs) and orthokeratinized odontogenic cyst (OOCs) show an indolent course, whilst others demonstrate an aggressive behavior and may have higher recurrence rate like odontogenic keratocysts (OKCs) and glandular odontogenic cysts (GOCs) [1,2,3].

OKC is a debatable odontogenic developmental cyst which has an aggressive behavior and high recurrence rates**.**It was reclassified as odontogenic cyst in the latest classification of head and neck tumors of the WHO, due to inadequate evidence to support its neoplastic origin [4].Additionally, OOC was originally defined as the uncommon orthokeratinized variant of OKC. However, recently WHO has designated OOC as a separate clinicopathologic entity**.** OOC appear to assume a dissimilar cell differentiation and exhibit a lower cellular activity than those of OKC[5].

Furthermore, DC surrounds an unerupted tooth and has a mechanism of expansion that is considered passive and its recurrence is rare. Additionally, GOC is a rare cyst, which shows a high rate of recurrence and a destructive potential [6].

A systematic review established an overall recurrence rate of OKC of about 25% after enucleation[7],while it is less than 2% after enucleation of OOC**.** Likewise, DC has very low recurrence rate. Dissimilarly, enucleation of GOC is associated with a high recurrence rate (30-50%) [8].

Epithelial-to-mesenchymal transition (EMT) is a critical step of tumorigenesis, where non-invasive and non-metastatic tumor cells lose their epithelial phenotype, attain invasive properties, infiltrate surrounding tissues and metastasize to secondary sites [9]. EMT is also recognized to contribute in the growth regulation of odontogenic cystic lesions and tumors [10].Evidence of the participation of the EMT in the progression of odontogenic lesions is inadequate, particularly in OKCs.

AEC: Amino ethyl carbazole, ANOVA: Analysis of variance ,DC: Dentigerous cyst, DAB: Diaminobenzidine, FAK: Focal adhesion kinase, GOC: Glandular odontogenic cyst, OKC:

Odontogenic keratocyst ,OOC: Orthokeratinized odontogenic cyst ,PCNA: Proliferating Cell Nuclear Antigen, PBS: Phosphate buffer solution, SD: Standard deviation

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that shows a crucial role in signal transductions and also shares in growth factor receptor signaling. Widespread studies designated that FAK is a significant mediator of cell adhesion, growth proliferation, survival, angiogenesis, and migration[11].

PCNA (Proliferating Cell Nuclear Antigen), is a nuclear protein which is critical for nucleic acid metabolism in DNA transcription and repair. It is expressed in high quantity in growing cells during cell cycle. It's mostly considered to be a marker of cell replication and also associated with DNA repair process and/or stimulation by growth factors [12,13].

Hence, this study will evaluate the differential immunohistochemical expression of FAK and PCNA by double immunostaining technique in OKC, OOC, DC and GOC. This will add to our knowledge about the role of EMT in invasion potential, proliferation and aggressiveness of these lesions that may help achieving the best treatment modality for the patients and lowering the recurrence rates.

Materials and methods:

Materials:

1. **Tissue samples:**
   Formalin fixed paraffin embedded "oral squamous cell carcinoma" was used as a positive control for FAK and "B- cell lymphoma" for PCNA. Negative control was prepared in the same method after omitting the step of the primary antibody application to ensure the specificity of the technique. Fixed paraffin embedded blocks of 40 specimens of OKC, OOC, DC and GOC (10 specimens of each cyst) were retrieved from the archives of the Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University. Intact tissue specimens, archival blocks from 2015 to 2020, uninflammed tissue specimens and primary lesions were included in our study whiledistorted tissue specimens, inflamed tissue specimens, archival blocks since more than five years and recurrent lesions were excluded.
2. **Antibodies and kits:**
   - FAK: Rabbit polyclonal anti-human FAK antibody 1 mg/ml in 0.01 M phosphate buffer solution (PBS), pH 7.4, containing 0.05% Proclin-300, 50% glycerol.*(Novus, SNF Medical- Egypt)*
   - PCNA: Rabbit polyclonal anti-human PCNA antibody1 mg/ml in PBS (without Mg2+ and Ca2+), pH 7.4, 150 mM NaCl, 0.02% sodium azide, 50% glycerol.*(Novus, SNF Medical- Egypt)*
   - Universal kit: The UltraView Universal Diaminobenzidine (DAB) Detection Kit. *(Dako, Life Trade Company- Egypt)*
   - Mouse/Rabbit PolyDetector AEC HRP Red Detection System. *(Novus, SNF Medical- Egypt)*

- Ultra V block.*(Thermo Fisher Scientific)*

3. **Manually prepared glycerin gel formula:** 1gm of gelatin and 0.1% of Thymol.

Methods:

1. **Glycerin gel mounting mediumpreparation:**

It was manually prepared by adding 1 gm of gelatin to 100 cc of boiling water and then mixed with 0.1 thymol, until it reached a viscous semi-fluid consistency.

2. **Histopathological preparation:**

From each block, two 4 μm thick sections were cut. One was mounted on a glass slide and stained by routine Hematoxylin and Eosin (H&E) stain to confirm the diagnosis. The other section was mounted on a positively charged slide.

3. **Double Immunohistochemical staining protocol:**

Double Immunostaining for FAK and PCNA was performed using Ventana Bench mark autostainer (USA) at Pathology Department, National Cancer Institute; Cairo University.

Immunohistochemical assessment:

Immunostained sections were examined using low and high power fields (x100, x200 and x400) by the light microscope. The positive immunoreactions for PCNA were detected by the DAB chromogen (nuclear brown color), while the positive immunoreactions for FAK were detected by the AEC chromogen (cytoplasmic red color) in an unstained background (no counterstain), the most homogenous areas of the reaction were chosen for evaluation.

The image analyzer computer system applying the software Leica Quin 500 (*Switzerland)* was used for the automated measuring of both (area percent of positive FAK and cell count of positive PCNA). It was performed in a standard frame area of $248x10^3$ μm^2 using magnification x200, five fields were measured per case.

Statistical analysis:

The obtained data from the image analysis for the studied groups was tabulated and presented as mean ± standard deviation (SD) values. The significance level was set as $P < 0.05$.

Statistical analysis was performed by using a computer program IBM SPSS. ANOVA test was used to compare more than 2 groups followed by Tukey's post Hoc test to compare between each two groups within the ANOVA test.

Results:

Histopathological examination of hematoxylin and eosin stained sections:

All examined cysts showed the typical H&E criteria of the epithelial lining and the connective tissue wall according toWHO classification of head and neck tumors [8]as shown in (Figures 1, 2,3,4,5,6,7,8&9).

Histopathological examination of double immunostained sections:

AllOKC lesions examined revealed positive cytoplasmic FAK expression in all layers of the cyst lining in all included cases which was noted to be intense, whereas positive nuclear PCNA expression was in all layers of the cyst lining in almost all included cases except few cases with

basal and parabasal expression (Figures 10&11), while all included OOC specimens revealed positive nuclear PCNA expression in a few number of cells especially in the basal one third, as well as positive cytoplasmic FAK expression in all layers of the epithelial lining of all tissue sections and it was noted to be faint (Figure 12)**,** additionally DC specimens revealed positive nuclear PCNA expression in a few number of cells which was noted to be faint, besides negative FAK expression in all included cases (Figure 13) **,**whereasall included cases of GOC revealed positive cytoplasmic FAK expression and nuclear PCNA expression in all layers of the cyst lining of all examined cases which was noted to be intense**.** The mucous cells were spared from both FAK and PCNA in cases where they were evident.Fibroblasts and endothelial cells showed positive expression of PCNA in few cells in the connective tissue wall in almost all studied cases of all studied cysts (Figure 14, 15, 16 &17).

Statistical analysis:

The greatest mean of PCNA nuclear count was recorded in GOC group, whereas the lowest value was the DC group. ANOVA test revealed that the difference between all groups was statistically significant. Tukey's post hoc test revealed a significant difference between each two groups (Table 1).

The greatest mean area percent of FAKimmunoexpressionwas recorded in OKC group, whereas the lowest value was recorded in DC group. ANOVA test revealed that the difference between all groups was statistically significant. Tukey's post hoc test revealed no significant difference between GOC and OKC groups (Table 2).

Discussion:

The majority of odontogenic cysts such as DCs and OOCs show an indolent course, whilst others demonstrate an aggressive behavior and may have higher recurrence rate like OKCs and GOCs)[1,2,3]. Consequently, there are divergent treatment approaches according to the contrasting behaviors and recurrence rates of those odontogenic cysts[8].EMT is recognized to contribute in the growth regulation of odontogenic cystic lesions and tumors[10].Evidence of the participation of the EMT in the progression of odontogenic lesions is inadequate, therefore we hypothesized that the current study may add to our knowledge about the role of EMT in invasion potential, proliferation and aggressiveness of these lesions that may help achieving the best treatment modality for the patients and lowering the recurrence rates.

FAK is a cytoplasmic tyrosine kinase that shows a crucial role in signal transductions and also shares in growth factor receptor signaling. Widespread studies designated that FAK is a significant mediator of cell adhesion, growth proliferation, survival, angiogenesis, and migration[11]. Besides, the expression of PCNA can be used as a cell proliferative marker. It's mostly considered to be a marker of cell replication and also associated with DNA repair process and/or stimulation by growth factor[12]. Consequently, we hypothesized that their different expression in diverse odontogenic cysts can clarify why OKC and GOC have different clinical behaviors, tendency of recurrence and proliferation abilities that varies from the other cysts.

To study the relationship between two antigens, multiple antigens can be localized using differently colored reaction products[14].Up to our knowledge double immunostaining technique has never been done using both FAK and PCNA in any lesion, so we studied the relationship between the two antigens ''PCNA and FAK'' labeled by two chromogens with two different colors ''DAB and AEC'' respectively. The studied antigens are involved in EMT and cell proliferation. Thus it was assumed that it might help us explore the divergent behaviors and recurrent rates of odontogenic cysts. Moreover, FAK is a cytoplasmic tyrosine kinase, while PCNA is a nuclear protein so both can be used in double IHC staining technique simultaneously according to the protocol assumed by (Loos, 2008) [14].

In the current study, the positive nuclear expression of PCNA in GOC was in all layers of the epithelial cyst lining of all examined cases. These findings suggest an increased proliferative activity even in the suprabasal layer of GOC indicating the aggressiveness of the cyst. This could also suggest increased growth factors and higher level of cytokines which stimulate epithelial cell proliferation directly and/or indirectly by inducing the secretion of some factors like keratinocyte growth factor with increased activity of keratin layers and tendency to recurrence [15].

Correspondingly, in OKC it was expressed in all layers of the cyst lining in almost all included cases just as GOC, whereas few cases showed positive expression only in the basal one third, while others in the basal two thirds, thus shows that OKC has inconstant proliferation activity. The basal cells normally have a proliferative role in the renewal of the epithelium; consequently the strictly basal staining pattern for PCNA is seen in normal and benign epithelial conditions [15].Therefore, when positive PCNA expression is seen in parabasal and superficial layers of the epithelium it indicates a high proliferation activity and an aggressive condition with a higher recurrence rate.

In addition, its expression in all included OOC cases was positive especially in the basal one third which indicates an indolent behavior and a less aggressive condition, while all DC specimens revealed very few positive cells which suggest a strictly benign behavior with the least destructive power.

Positive PCNA expression in fibroblasts and some endothelial cells in the connective tissue wall in all studied cysts suggesting that these cell types might form a cellular network sharing regulation by the stimulatory signals promoting cyst growth, angiogenesis or as an inflammatory reaction [16].Besides**,** negative staining of the mucous cells by both FAK and PCNA in cases of GOC may suggest that they do not share in the proliferation activity of GOC.

Several studies were done to compare the expression of PCNA in OKC with other odontogenic cyst [12,13, 17].One studyclaimed that PCNA showed 100% expression in OKC compared to 60% in radicular cyst. These findings may explain its different clinical behaviors and the tendency of recurrence [13]**.** Another study was done on the expression of PCNA on DC, radicular cyst, OKC and ameloblastoma which concluded that among the cysts OKC has higher cellular kinetics in comparison to DC and radicular cyst, which propose that OKC has intrinsic growth potential, similar to ameloblastoma [18].

Only one study was done to compare the expression PCNA in GOC with low-grade MEC, and radicular cyst with mucous metaplasia and there were no significant differences between groups in the expression of PCNAwhich was surprising and unexplained as the three studied lesions show an extremely different behavior, treatment modalities as well as varying recurrence rates when compared to each other. [19]. Up to our knowledge there no studies were done on the expression of PCNA on OOC.

Accordingly, GOC has the highest proliferating activity, DNA stimulation by growth factors and cell replication followed by OKC which is consistent with their aggressive behavior and high recurrence rates when compared with the indolent OOC and DC [12].

Furthermore, regarding FAK cytoplasmic expression, it was positive in the whole epithelial thickness of OKC and GOC in most of the cells thus indicates a high EMT in both cysts, surprisingly in OOC it was positive in the whole epithelial thickness but it was noted to be faint. Which is possibly be explained by the fact that EMT may be expressed at different stages of cyst growth, as there was a difference in the intensity of the staining. Unfortunately, there were no previous studies on EMT in OOC, but positive immunostaining was also detected in benign epithelial hyperplasias and pre-invasive dysplastic lesions, indicating that FAK overexpression is an early event during tumorigenesis[20].On the other hand it was totally negative in all examined cases of DC thus suggests the entirely indolent behavior of this cyst with no EMT activity.

Only one study compared between the expression of FAK in OKC, DC and OOC. There was strong FAK expression in OKC and negative/weak expression in DC and OOC, suggesting that FAK could be responsible for aggressive behavior, local invasiveness, and proliferation in OKC [20]. Up to our knowledge the expression of FAK on GOC has never been studied.

Accordingly, OKC has the highest invasion potential, proliferation and aggressiveness as FAK is a significant mediator of cell adhesion, growth proliferation, survival, angiogenesis, and migration[11]. OKC also has the highest EMT ability as the expression of FAK leads to the upregulation of EMT markers, including vimentin and Snail[21].This isfollowed by GOC which has no significant difference when its FAK expression was compared to OKC. This is consistent with the aggressive behavior and high recurrence rates of both OKC and GOC when compared to the indolent OOC and DC.

In conclusion, OKC has the highest EMT ability followed by GOC, while GOC has the highest proliferation activity followed by OKC. On the other hand, OOC and DC have the lowest EMT ability and proliferation activity, which can explain the diverse behavior of those odontogenic cysts. Follow up after GOC and OKC treatment is a must.

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Conflict of Interest Statement:

Authors declare no conflict of interest.

Figure legends:

Figure (1). Photomicrograph of an H&E stained section of DC demonstrating a cystic cavity lined by thin and regular stratified squamous epithelium of about two to four cells in thickness. The connective tissue wall is loose and uninflamed, composed of blood vessels, fibroblasts and collagen bundles. (Magnification x100)

Figure (2). Photomicrograph of an H&E stained section of OOC demonstrating a cystic cavity filled with orthokeratin (black arrow) and a prominent granular cell layer (blue arrow). (Magnification x200)

Figure (3). Photomicrograph of an H&E stained section of OKC demonstrating a cyst lining composed of a uniform thin corrugated parakeratinized stratified squamous epithelium. (Magnification x100)

Figure (4). Photomicrograph of an H&E stained section of OKC demonstrating the main cyst wall (blue arrow) and a daughter cyst of odontogenic epithelium within the cyst wall (black arrow). The connective tissue wall composed of blood vessels, fibroblasts and collagen bundles. (Magnification x100)

Figure (5). Photomicrograph of an H&E stained section of GOC demonstrating a cyst lining composed of non-keratinized stratified squamous epithelium of varying thickness. A microcyst is also observed (black arrow). (Magnification x100)

Figure (6). Photomicrograph of an H&E stained section of GOC demonstrating the superficial layer of the epithelium which shows eosinophilic cuboidal cells (hobnail cells) (black arrows) and clear cells (blue arrows). (Magnification x200)

Figure (7). Photomicrograph of an H&E stained section of GOC demonstrating the superficial layer of the epithelium which shows basophilic mucous cells (black arrows). (Magnification x200)

Figure (8). Photomicrograph of an H&E stained section of GOC demonstrating the superficial layer of the epithelium which shows tufting (black arrows). (Magnification x200)

Figure (9). Photomicrograph of an H&E stained section of GOC demonstrating a daughter cyst of odontogenic epithelium within the fibrous wall and connected to the overlying epithelium (black arrow). (Magnification x100)

Figure (10). Photomicrograph of a double immunostained section of DC demonstrating thin epithelial cyst lining showing positive nuclear PCNA expression (brown color) in few cells and negative FAK expression. Positive PCNA expression in fibroblasts in the connective tissue wall was also observed. (Magnification x200)

Figure (11). Photomicrograph of a double immunostained section of OOC demonstrating epithelial cyst lining showing positive nuclear PCNA expression (brown color) especially in the basal one third, as well as a positive cytoplasmic FAK expression (red color) in all layers of the epithelial lining. (Magnification x200)

Figure (12). Photomicrograph of a double immunostained section of OKC demonstrating a positive nuclear PCNA expression (brown color), as well as a positive cytoplasmic FAK expression (red color) in all layers of the cyst lining. PCNA expression in fibroblasts and endothelial cells in the connective tissue wall was also observed. (Magnification x100)

Figure (13). Photomicrograph of a double immunostained section of OKC demonstrating epithelial cyst lining with numerous areas of budding, showing a positive nuclear PCNA expression (brown color), as well as a positive cytoplasmic FAK expression (red color) in all layers of the epithelial lining. (Magnification x200)

Figure (14). Photomicrograph of a double immunostained section of GOC demonstrating a positive nuclear PCNA expression (brown color) in most of the cells, as well as a positive cytoplasmic FAK expression (red color) in all layers of the cyst lining. PCNA expression in fibroblasts and endothelial cells in the connective tissue wall was also observed. (Magnification x100)

Figure (15). Photomicrograph of a double immunostained section of GOC demonstrating a positive nuclear PCNA expression (brown color), as well as a positive cytoplasmic FAK expression (red color) in all layers of the cyst lining. PCNA expression in fibroblasts and endothelial cells in the connective tissue wall was also observed. Note the connective tissue arcade (blue arrow). (Magnification x200)

Figure (16). Photomicrograph of a double immunostained section of GOC demonstrating a positive nuclear PCNA expression (brown color), as well as a positive cytoplasmic FAK expression (red color) in all layers of the cyst lining. Note the sparing in the mucous cells (blue arrow). (Magnification x200)

Figure (17). A higher magnification demonstrating positive nuclear PCNA expression (brown color), as well as positive cytoplasmic FAK expression (red color) in all layers of the epithelial cyst lining. (Magnification x400)

Tables:

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

P.O.C	OOC	GOC	DC	OKC
Mean	21.8^{a}	75^{b}	8^{c}	31.6^{d}
Std Dev	4.31	7.35	2	6.65
Std error	2.15	3.67	1	3.33
Max	28	84	11	44
Min	15	65	5	25
F value	138.583			
P Value	<0.0001 *significant at p<0.05			

Table (2) Area percent of FAK immunoexpression in all groups and significance of the difference using (ANOVA) test

P.O.C	OOC	GOC	DC	OKC
Mean	3.91^{a}	17.64^{b}	0.73^{c}	23.18^{b}
Std Dev	2.26	6.11	0.27	3.95
Std error	1.01	2.73	0.12	1.77
Max	7.30	26.85	1.15	26.94

Min	1.14	7.63	0.40	15.94
F value				39.881
P Value				0.001* significant at p<0.05

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