FAK and PCNA Double Immunohistochemical Expression among Different Oral Squamous Cell Carcinoma Grades

Short running title: FAK and PCNA Double Immunostaining in OSCC

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Abstract:
Objective:
Focal adhesion kinase (FAK) is a tyrosine kinase that belongs to the focal adhesion complex that proved to have a serious role in cancer progression through its upregulation, while proliferating cell nuclear antigen (PCNA) is a proliferation indicating marker that involved in tumors behavior regarding invasiveness. Our aim is to assess the synchronization in the upregualtion of both markers through double immunostaining and their correlation with prognosis worsening in well differentiated and poorly differentiated oral squamous cell carcinoma (OSCC).

Study design:
A total number of 60 OSCC lesions distributed equally (n= 30) between the first group (well differentiated OSCC) & second group of (poorly differentiated OSCC). Double immunostaining with FAK & PCNA was carried out to determine their localization. Area percent measurement of FAK and nuclear count for PCNA were performed and analyzed.

Results:
Both area percent measurement of FAK and nuclear count of PCNA are higher in the poorly differentiated OSCC group than that of the well differentiated OSCC group, results showed statistically significant difference between the groups.

Conclusion:
Our results indicate that both FAK and PCNA are upregulated in poorly differentiated OSCC than in the well differentiated OSCC supporting their role in cancer aggressiveness & progression together with poor prognosis in higher grades of same tumor.

Keywords: Double immunostaining; FAK; PCNA, OSCC

Introduction:
Cancer is considered the second mortality cause among the top ten causes of mortality (1). Although oral cancer isn’t among top ten cancers according to its incidence recorded by the Globocan observatory but it still affecting
large number of adults worldwide where 529,708 new cases were diagnosed last year with oral and oropharyngeal cancers including salivary gland malignancies, where the lip and oral cancer is the 17th among all cancers classified by site, where it caused 177,757 deaths last year of (2).

Oral squamous cell carcinoma is the malignancy aroused from squamous epithelial cells constituting the oral cavity it accounts for over 90% of oral cancers (3) and ranked as the 6th most common cancer worldwide with all its grades rendering it an alarming heath issue(4).

OSCC has different stages and gradings that must be determined a priori to treatment planning as it will greatly impact not only the treatment with adjunctive modalities after the surgical procedure but also the future prognosis (5).

Despite the proposal of multiple complicated systems for OSCC grading but still the world health organization only approved Broders’ classification which is the simple one of being classified as well, moderate and poorly differentiated(6).

Although H& E stained sections are the standard pathological diagnostic approach but immunohistochemical staining is considered of great benefit especially in evaluating other prognostic factors as tumor microenvironment changes, epithelial to mesenchymal transition helping for further carcinomas metastasis and tumor progression as well (7).

FAK is a tyrosine kinase protein found in cytoplasm its expression is common among different types of cancers it acts mainly on the host immune response to tumor cells (8) and the epithelial to mesenchymal transition (EMT) thus helping cellular migration through various mechanisms, it can also help in cancer cell stemness so its expression is considered as an alarm for tumor rapid growth, invasion and future metastasis (9).

PCNA is one of the cell cycle specific antigens whose detection predicts high proliferative activity of the tumor and its upregulation used as a helpful prognostic marker (11) several studies find its expression is correlated to the OSCC histopathological grading where it is over expressed in high grade lesions and reduced in low grade ones and the difference was with significance (12, 13)

Other study finds that targeting PCNA by downregulating it leads to losing capacity of OSCC cells for migration, invasion and proliferation (14),thus further strong evidence availability for FAK and PCNA double immunohistochemical expression in OSCC and their use as validated prognostic markers may help in new target therapy development as tyrosine kinase inhibitors used in breast carcinomas treatment lapatinib (10) and give the opportunity to think about new treatment modalities to save patients lives.

Materials & Methods

Tissue samples collection & sorting:
A comparative invitro study was performed on formalin- fixed paraffin -embedded blocks of 60 specimens of OSCC (30 well differentiated & 30 poorly differentiated lesions) Sorting out OSCC tissue samples was carried out according to predetermined eligibility criteria for both study groups samples: a) Intact tissue specimens, b) Archival blocks from 2015 to 2020, c) un inflammed tissue specimens d) Primary non recurrent lesions e) well and poorly differentiated OSCC then all included tissue samples were reviewed to be classified according the latest WHO classification (6), all tissue samples were obtained from the archives of Oral & Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University, Hematoxylin and Eosin stained the reviewed by two pathologists other than the authors for diagnosis confirmation before preparing the tissue sections for immunohistochemical staining.
Tissue Samples Preparation & Staining:
All formalin-fixed paraffin-embedded blocks of 60 specimens of OSCC with both grades were sectioned in 4 μm thick sections and deparaffinized for double immunostaining procedures with both anti-FAK antibody (Novus Biologicals, Colorad, USA) and anti-PCNA antibody (Agilent Dako, California, USA). Double immunostaining was performed according to the manufacturer’s instructions at dilution 1:100 using (Ventana BenchMark autostainer, Arizona, USA) at Pathology Department, National Cancer Institute; Cairo University. The positive immunoreactions for FAK were detected by the AEC chromogen (cytoplasmic red color) (Novus Biologicals, Colorad, USA) in an unstained background while for PCNA the DAB chromogen detection kit (thermo fisher scientific, Massachusetts, USA) was used, in addition Formalin fixed paraffin embedded “human spleen tissue” was used as a positive control for FAK while “B-cell lymphoma” was used PCNA.

Immunohistochemical Assessment & Image Analysis:
The positive immunoreactions of PCNA is represented by its nuclear brown color presentation, while the positive immunoreactions for FAK were detected by its cytoplasmic red color presentation in an unstained background with the most homogenous areas of the reaction chosen for evaluation and were assessed by both a) Transmission light microscope (Leica model DM LB2, Switzerland) to assess the positivity of the immunoreactions of FAK & PCNA at low & high power magnifications. b) Image analyzer computer system with Leica Quin 500 software (Leica Microsystems, Switzerland) was used for the automated measuring of both area percent of positive FAK and cell count of positive PCNA using standard frame area of 248x10^3 μm^2 with 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 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp)

Results:
- **Expression of FAK & PCNA in double immunostained well differentiated OSCC sections:**
  The whole cases of well differentiated OSCC (n=30) either show weak or negative nuclear immunoreactivity of PCNA as well as weak cytoplasmic to moderate FAK expression with decreased area percent for both markers as shown in figure (1,2).

- **Expression of FAK & PCNA in double immunostained poorly differentiated OSCC sections:**
  The whole cases of poorly differentiated OSCC (n=30) show strong nuclear positive immunoreactivity of PCNA as well as strong cytoplasmic FAK expression with increased area percent for both markers as shown in figure (3,4).

By comparison between both groups well differentiated OSCC and poorly differentiated OSCC it was found that poorly differentiated OSCC group revealed a higher mean (138.73) for PCNA nuclear count compared to the mean value of Well-differentiated SCC group (93.73). Unpaired t-test revealed that the difference was statistically significant (P <0.0001). (Table1, Fig.5), as well as for FAK; poorly differentiated SCC group revealed a higher mean (55.39) area percent compared to the mean value (7.06) of Well-differentiated SCC group. Unpaired t-test revealed that the difference was statistically significant (P <0.0001), (Table2, Fig.6)
Discussion:
OSCC is among top 10 malignancies affecting cancer patients worldwide and despite being an alarming public health issue there is no satisfactory well documented immunohistochemical tissue markers for prognosis. So our aim was to investigate the potential use of double immunostaining technique with FAK &PCNA molecules to evaluate the prognosis of those lesions, this was influenced by the evidence of their expression in aggressive and invasive lesions(15),(16). Furthermore many pharmacological preclinical (17), (18),(19) and clinical trials (20,21,22)were conducted to target FAK as anticancer therapy, but neither preclinical or clinical trials were conducted on OSCC. PCNA was a target for anticancer therapy trials as well(23,24). Thus the decision to study those markers in tumors related to the head and neck area as this may help in developing either solid evidence to use such markers as a routine examination for assessment of OSCC and for developing an anticancer therapy.

The current study results showed strong positivity for both PCNA & FAK markers among poorly differentiated OSCC, being poorly differentiated means it have a worse prognosis. While same markers were showing weak positivity among well differentiated OSCC group .this was found in accordance with shelaam et al (13)that found PCNA have higher expression in poorly differentiated and moderately differentiated SCC and less expression in well differentiated lesions. In addition to shelaam et al compared PCNA expression in SCC with different potentially malignant lesions and found SCC have the highest expression and the difference was statistically significant this can be explained that as the cells lose their differentiation it have higher expression of PCNA as it help in epithelial to mesenchymal transition process that help in tumor progression and metastasis (25)

On the other hand, some studies showed results similar to our study regarding PCNA expression such as Rüdiger G Steinbeck et al, where PCNA was noticed focally expressed in highly differentiated squamous cell carcinoma while it was seen in the entire tumor mass of poorly differentiated squamous cell carcinoma (26) poosarla et al found 3 to 4 fold increase in PCNA values in different grades of oral squamous cell carcinoma where the difference in PCNA expression found to be increasing with increasing grade of malignancy of the oral squamous cell carcinoma (12).

Regarding FAK expression our current study also showed increased expression of it with increasing the tumor grade, so high expression of FAK was found in poorly differentiated OSCC while it was weakly expressed in well differentiated lesions. Our results are consistent with Kato et al where they found FAK is upregulated with higher grad OSCC. Moreover they found strong correlation between poor clinical stage of the tumor and the FAK upregulation and this is found to be in accordance of our suggestion that FAK is highly detected in metastatic lesions, lesions showing recurrence as well as lesions with poor prognosis (27).

Murphy et al, mentioned in their study that many epithelial cancers including breast, colorectal and lung cancer showed high FAK expression that associated with poor prognosis (8) that is our main suggestion to use FAK as a prognostic marker and this may be explained as a consequence to FAK role in EMT and cancer cell stemness(8)

Xia J et al, also document their finding that FAK expression show upregulation from normal mucosa, dysplastic lesions and then among OSCC grades itself and they explained that by FAK have role in carcinogenesis thus it showed that sequential increase (28)

De Vicente et al conducted a study on oral dysplasia and OSCC and found that FAK expression is increasingly strong from oral dysplastic lesions to OSCC with the highest expression in worst grades that is the same as both our suggestion and results (29)
Conclusion:
PCNA and FAK expression were significantly associated with increased features of malignancy in OSCC in the form of Strong coexpression of both proteins in poorly differentiated OSCC group than that of well differentiated OSCC group reflected a good prognostic value of using both markers together.

Funding:
This research had no funding.

Conflict of Interest Statement:
Authors declare no conflict of interest.

References:


Figures:

![Photomicrograph of double immunostained section of well differentiated OSCC showing positive cytoplasmic FAK expression and negative PCNA expression. (Magnification x 200)](image-url)
Figure (2). Photomicrograph of double immunostained section of well differentiated OSCC showing an epithelial island demonstrating positive cytoplasmic FAK expression and negative PCNA expression. (Magnification x 200)

Figure (3). Photomicrograph of double immunostained section of poorly differentiated OSCC showing OSCC showing strong FAK cytoplasmic expression and strong nuclear PCNA expression. (Magnification x 200)
Figure (4). Photomicrograph of double immunostained section of poorly differentiated OSCC showing strong FAK cytoplasmic expression and strong nuclear PCNA expression. (Magnification x 400)

Figure (5). Column chart showing mean PCNA nuclear count of well differentiated SCC group and poorly differentiated SCC group.

Mean

PCNA nuclear count

Well-differentiated SCC Poorly differentiated SCC

95,3 137,5
Figure(6). Column chart showing mean FAK area percent of well differentiated SCC group and poorly differentiated SCC group.

**Results**

**I-PCNA nuclear count**

Table (1) PCNA nuclear count of well differentiated SCC group vs poorly differentiated SCC group, and significance of the difference using unpaired t-test.

<table>
<thead>
<tr>
<th>P.O.C</th>
<th>Well-differentiated SCC</th>
<th>Poorly differentiated SCC</th>
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<tbody>
<tr>
<td>Mean</td>
<td>93.73</td>
<td>138.73</td>
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<tr>
<td>Std Dev</td>
<td>17.32</td>
<td>21.04</td>
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<tr>
<td>Std Error</td>
<td>3.22</td>
<td>3.90</td>
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<tr>
<td>Max</td>
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<td>189</td>
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<tr>
<td>Min</td>
<td>45</td>
<td>109</td>
</tr>
<tr>
<td>t-value</td>
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<td>9.0443</td>
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<tr>
<td>P-value</td>
<td></td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

*significant at p<0.05

**II-FAK area percent**

Table (2) FAK nuclear count of well differentiated SCC group vs poorly differentiated SCC group, and significance of the difference using unpaired t-test.
<table>
<thead>
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<th>P.O.C</th>
<th>Well-differentiated SCC</th>
<th>Poorly differentiated SCC</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.06</td>
<td>55.39</td>
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<tr>
<td>Std Dev</td>
<td>2.38</td>
<td>12.03</td>
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<tr>
<td>Std Error</td>
<td>0.44</td>
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<tr>
<td>Max</td>
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<td>Min</td>
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<td>t-value</td>
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<tr>
<td>P-value</td>
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</tbody>
</table>

*significant at p<0.05