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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 histoplathological 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 deparrafinized 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 conductued 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.

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Conflict of Interest Statement:

Authors declare no conflict of interest.

References:

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016 Jan;66(1):7-30.

2) World Health Organization: Globocan 2020. Cancer Today. Data visualization tools for exploring the global cancer burden in 2020. https://gco.iarc.fr/today/data/factsheets/cancers/1-Lip-oral-cavity-fact-sheet.pdf, accessed February 2021

3) Jiang X, Wu J, Wang J, Huang R. Tobacco and oral squamous cell carcinoma: A review of carcinogenic pathways. Tobacco induced diseases. 2019;17.

4) Suresh GM, Koppad R, Prakash BV, Sabitha KS, Dhara PS. Prognostic indicators of oral squamous cell carcinoma. Annals of maxillofacial surgery. 2019 Jul;9(2):364.

5) Almangush A, Mäkitie AA, Triantafyllou A, de Bree R, Strojan P, Rinaldo A, Hernandez-Prera JC, Suárez C, Kowalski LP, Ferlito A, Leivo I. Staging and grading of oral squamous cell carcinoma: An update. Oral Oncology. 2020 Aug 1;107:104799.

6) El-Naggar AK, Chan JK, Grandis JR, Takata T, Slootweg PJ, editors. WHO classification of head and neck tumours. International Agency for Research on Cancer (IARC); 2017.

7) Woolgar JA, Triantafyllou A. Pitfalls and procedures in the histopathological diagnosis of oral and oropharyngeal squamous cell carcinoma and a review of the role of pathology in prognosis. Oral oncology. 2009 Apr 1;45(4-5):361-85.

8) Murphy JM, Rodriguez YA, Jeong K, Ahn EY, Lim ST. Targeting focal adhesion kinase in cancer cells and the tumor microenvironment. Experimental & Molecular Medicine. 2020 Jun 9:1-0.

9) Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. Nature reviews cancer. 2014 Sep;14(9):598-610.

10) Cervino G, Fiorillo L, Herford AS, Romeo U, Bianchi A, Crimi S, D'Amico C, De Stefano R, Troiano G, Santoro R, Laino L. Molecular biomarkers related to oral carcinoma: clinical trial outcome evaluation in a literature review. Disease markers. 2019 Mar 25;2019.

11) Jurikova M, Danihel Ľ, Polák Š, Varga I. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. Acta histochemica. 2016 Jun 1;118(5):544-52.

12) Chandrashekar Poosarla MR, Ramesh K, Gudiseva S, Bala S, Sundar M. Proliferating cell nuclear antigen in premalignancy and oral squamous cell carcinoma. Journal of clinical and diagnostic research: JCDR. 2015 Jun;9(6):ZC39.

13) Sheelam S, Reddy SP, Kulkarni PG, Nandan SR, Keerthi M, Raj GS. Role of cell proliferation and vascularity in malignant transformation of potentially malignant disorders. Journal of oral and maxillofacial pathology: JOMFP. 2018 May;22(2):281.

14) Kong Y, Feng Y, Xiao YY, Liu SC, Li XG, Yang QL, Chu WH, Liu JG. LncRNA LUCAT1 promotes rowth, migration, and invasion of oral squamous cell carcinoma by upregulating PCNA. Eur Rev Med Pharmacol Sci. 2019 Jun 1;23(11):4770-6

15) Lu EM, Ratnayake J, Rich AM. Assessment of proliferating cell nuclear antigen (PCNA) expression at the invading front of oral squamous cell carcinoma. BMC oral health. 2019 Dec;19(1):1-7.

16)Thiagarajan PS, Sinyuk M, Turaga SM, Mulkearns-Hubert EE, Hale JS, Rao V, Demelash A, Saygin C, China A, Alban TJ, Hitomi M. Cx26 drives self-renewal in triple-negative breast cancer via interaction with NANOG and focal adhesion kinase. Nature communications. 2018 Feb 8;9(1):1-4.

17) Lee BY, Timpson P, Horvath LG, Daly RJ. FAK signaling in human cancer as a target for therapeutics. Pharmacology & therapeutics. 2015 Feb 1;146:132-49.

18) Osterman CJ, Ozmadenci D, Kleinschmidt EG, Taylor KN, Barrie AM, Jiang S, Bean LM, Sulzmaier FJ, Jean C, Tancioni I, Anderson K. FAK activity sustains intrinsic and acquired ovarian cancer resistance to platinum chemotherapy. Elife. 2019 Sep 3;8:e47327.

19) Jiang H, Hegde S, Knolhoff BL, Zhu Y, Herndon JM, Meyer MA, Nywening TM, Hawkins WG, Shapiro IM, Weaver DT, Pachter JA. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nature medicine. 2016 Aug;22(8):851-60.

20) Brown NF, Williams M, Arkenau HT, Fleming RA, Tolson J, Yan L, Zhang J, Singh R, Auger KR, Lenox L, Cox D. A study of the focal adhesion kinase inhibitor GSK2256098 in patients with recurrent glioblastoma with evaluation of tumor penetration of [11C] GSK2256098. Neuro-oncology. 2018 Nov 12;20(12):1634-42.

21) Mak G, Soria JC, Blagden SP, Plummer R, Fleming RA, Nebot N, Zhang J, Mazumdar J, Rogan D, Gazzah A, Rizzuto I. A phase Ib dose-finding, pharmacokinetic study of the focal adhesion kinase inhibitor GSK2256098 and trametinib in patients with advanced solid tumours. British journal of cancer. 2019 May;120(10):975-81.

22) Shimizu T, Fukuoka K, Takeda M, Iwasa T, Yoshida T, Horobin J, Keegan M, Vaickus L, Chavan A, Padval M, Nakagawa K. A first-in-Asian phase 1 study to evaluate safety, pharmacokinetics and clinical activity of VS-6063, a focal adhesion kinase (FAK) inhibitor in Japanese patients with advanced solid tumors. Cancer chemotherapy and pharmacology. 2016 May;77(5):997-1003.

23) Gu L, Lingeman R, Yakushijin F, Sun E, Cui Q, Chao J, Hu W, Li H, Hickey RJ, Stark JM, Yuan YC. The anticancer activity of a first-in-class small-molecule targeting PCNA. Clinical Cancer Research. 2018 Dec 1;24(23):6053-65.

24) Li P, Wang Q, Wang H. MicroRNA-204 inhibits the proliferation, migration and invasion of human lung cancer cells by targeting PCNA-1 and inhibits tumor growth in vivo. International journal of molecular medicine. 2019 Mar 1;43(3):1149-56.

25) Peng B, Ortega J, Gu L, Chang Z, Li GM. Phosphorylation of proliferating cell nuclear antigen promotes cancer progression by activating the ATM/Akt/GSK3β/Snail signaling pathway. Journal of Biological Chemistry. 2019 Apr 26;294(17):7037-45.

26) Steinbeck RG, Moege J, Heselmeyer KM, Klebe W, Neugebauer W, Borg B, Auer GU. DNA content and PCNA immunoreactivity in oral precancerous and cancerous lesions. European Journal of Cancer Part B: Oral Oncology. 1993 Jan 1;29(4):279-84.

27) Kato A, Kato K, Miyazawa H, Kobayashi H, Noguchi N, Kawashiri S. Focal Adhesion Kinase (FAK) Overexpression and Phosphorylation in Oral Squamous Cell Carcinoma and their Clinicopathological Significance. Pathology & Oncology Research. 2019 Sep 14:1-9.

28) Xia J, Lv N, Hong Y, Li C, Tao X, Chen X, Cheng B. Increased expression of focal adhesion kinase correlates with cellular proliferation and apoptosis during 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis. Journal of oral pathology & medicine. 2009 Jul;38(6):524-9.

29) de Vicente JC, Rodrigo JP, Rodriguez-Santamarta T, Lequerica-Fernández P, Allonca E, García-Pedrero JM. Cortactin and focal adhesion kinase as predictors of cancer risk in patients with premalignant oral epithelial lesions. Oral oncology. 2012 Jul 1;48(7):641-6.

Figures:

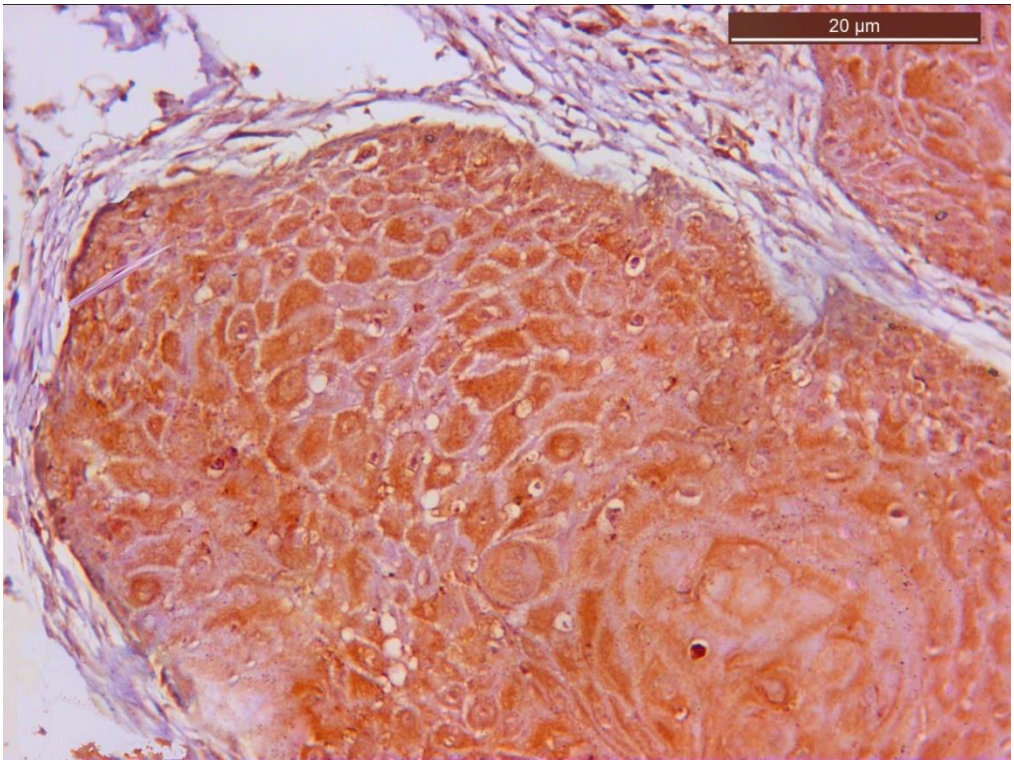


Figure (1). Photomicrograph of double immunostained section of well differentiated OSCC showing positive cytoplasmic FAK expression and negative PCNA expression. (Magnification x 200)

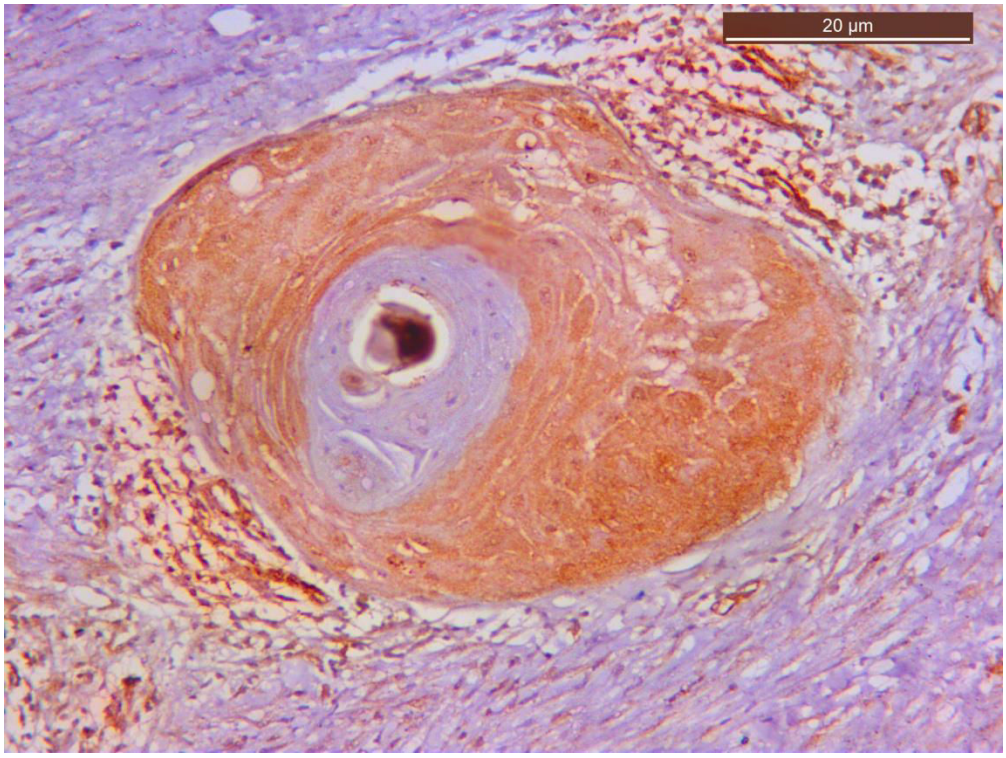


Figure (2). Photomicrograph of doubleimmunostained section of well differentiated OSCC showing an epithelial island demonstrating positive cytoplasmic FAK expression and negative PCNA expression. (Magnification x 200)

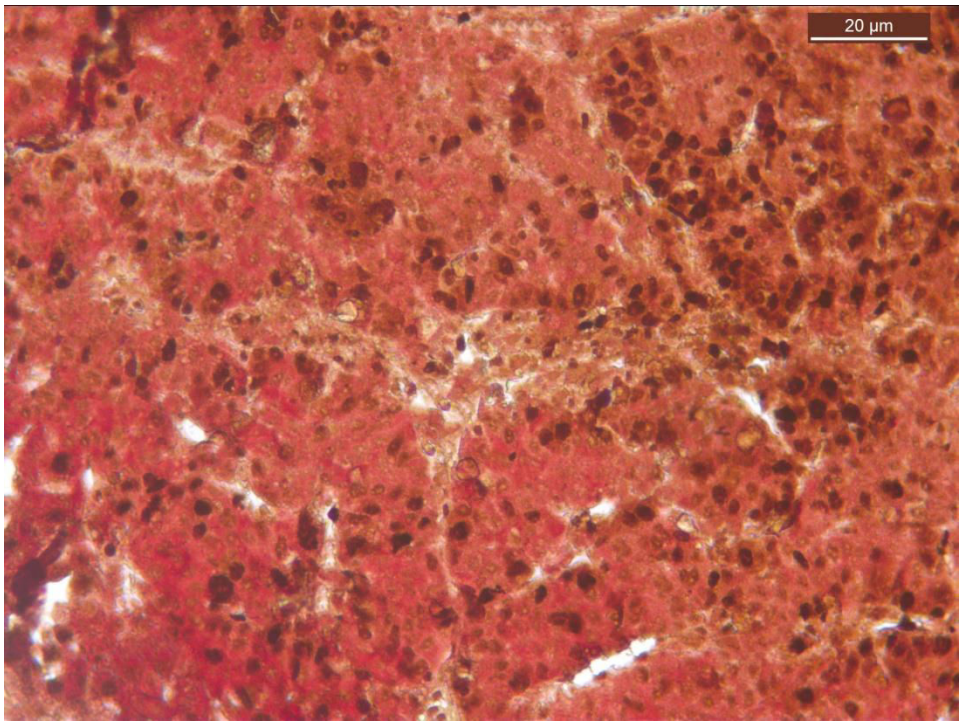


Figure (3). Photomicrograph of doubleimmunostained section of poorly differentiated OSCC showing OSCC showing strong FAK cytoplasmic expression and strong nuclear PCNA expression. (Magnification x 200)

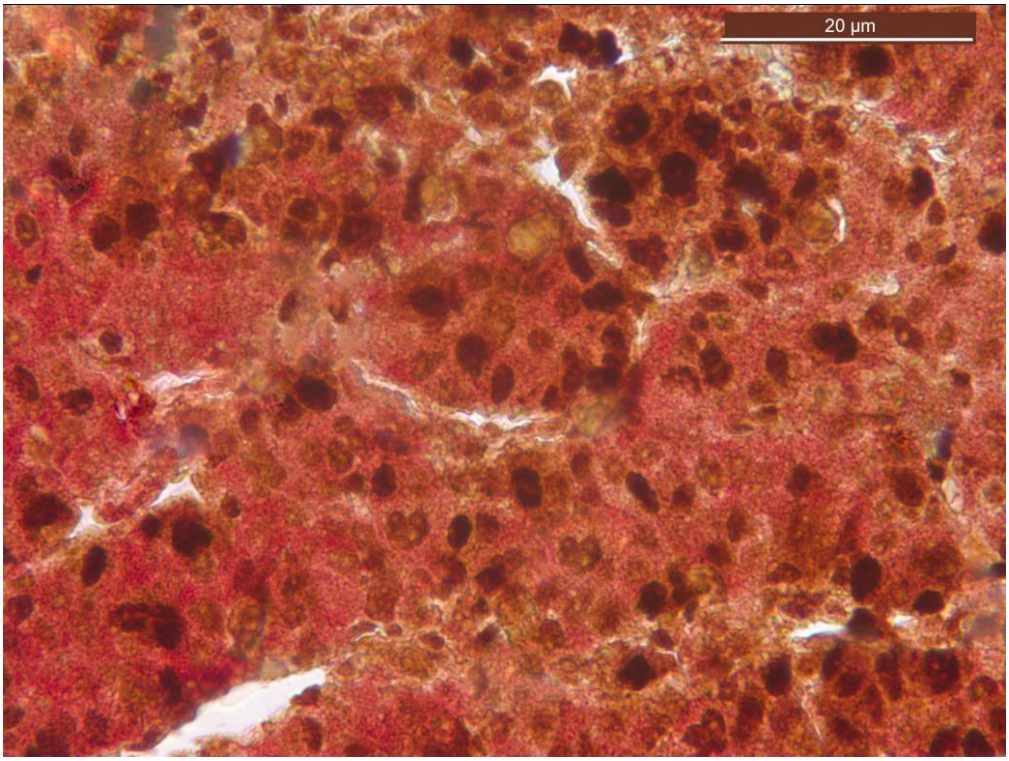


Figure (4). Photomicrograph of double immunostained section of poorly differentiated OSCC showing strong FAK cytoplasmic expression and strong nuclear PCNA expression. (Magnification x 400)

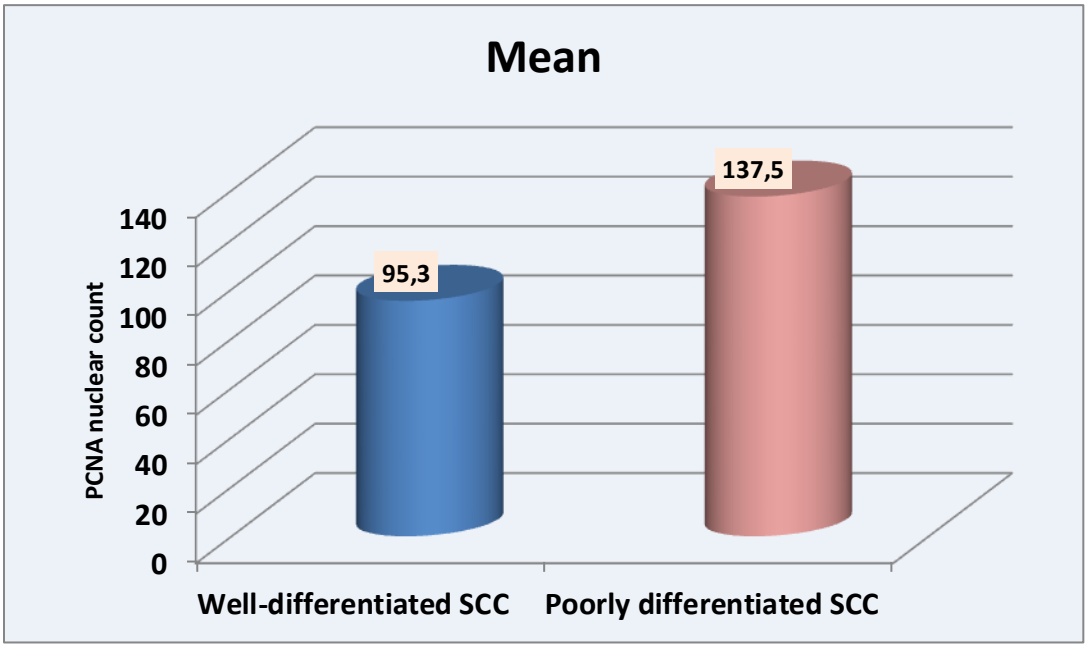
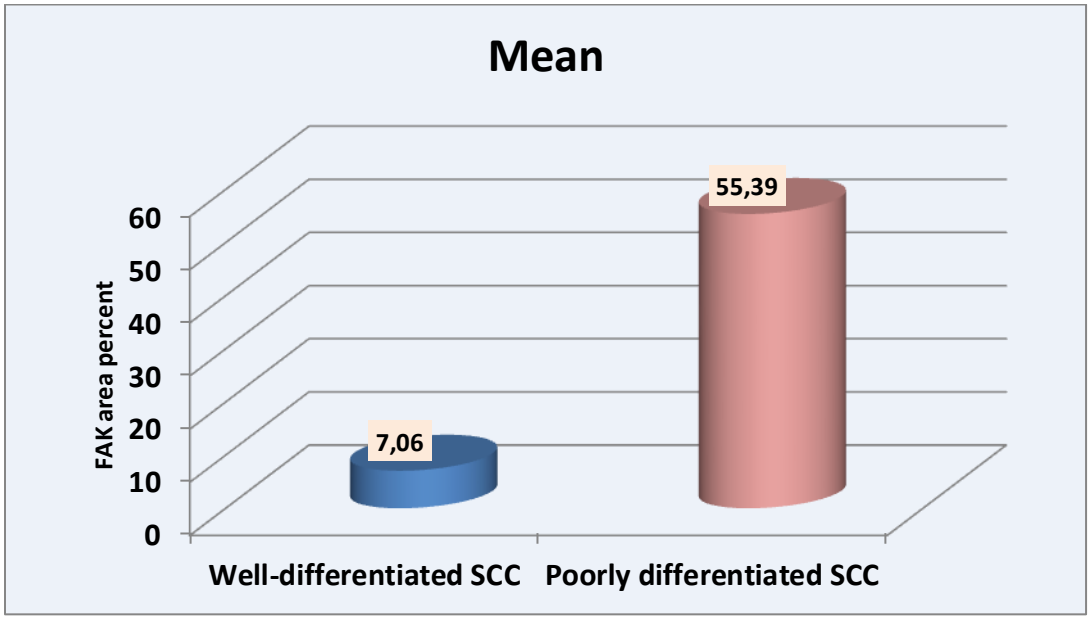


Figure (5). Column chart showing mean PCNA nuclear count of well differentiated SCC group and poorly differentiated SCC group.



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.

P.O.C	Well-differentiated SCC	Poorly differentiated SCC
Mean	93.73	138.73
Std Dev	17.32	21.04
Std Error	3.22	3.90
Max	121	189
Min	45	109
t-value	9.0443	
P-value	<0.0001*	

*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.

P.O.C	Well-differentiated SCC	Poorly differentiated SCC
Mean	7.06	55.39
Std Dev	2.38	12.03
Std Error	0.44	2.46
Max	12.32	78.72
Min	3.11	32.06
t-value	21.5861	
P-value	<0.0001*	

*significant at $p<0.05$