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Immunohistochemical characterization of podoplanin as a biomarker in speckled leukoplakia and oral squamous cell carcinoma

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Abstract

Background: Podoplanin is a small mucin-like transmembrane protein and lymphatic endothelial marker and is highly expressed into oral cancer and some dysplastic oral premalignant lesion.

Aims and objectives: Aim of the study is to determine the role of Podoplanin as a biomarker in oral tumorigenesis in patients having speckled leukoplakia and oral squamous cell carcinoma (OSCC) and to compare grades of dysplasia in the speckled leukoplakia with OSCC by podoplanin expression for cancer risk.

Materials and methods: In the present study, total 30 patients were included. From them, 15 patients were of speckled leukoplakia while 15 patients were of OSCC. 3-4µm sections were immunohistochemically stained with antibody against Podoplanin to determine the expression and staining intensity of podoplanin in the epithelium of speckled leukoplakia and OSCC and statistically analysed.

Results: By applying chi square test, Podoplanin expression in patients with speckled leukoplakia having mild dysplasia showed low risk while in moderate and severe dysplasia showed high risk which is statistically significant (p value 0.02). Patients with Well differentiated squamous cell carcinoma showed weak reactivity and moderately differentiated squamous cell carcinoma showed high reactivity which was statistically significant (p value 0.013).

Conclusion: Podoplanin expression in premalignant lesion increases with increasing grades of dysplasia which suggest high risk of transformation into OSCC. Thus,

Podoplanin can be used as a biomarker for early oral tumorigenesis and for malignant transformation risk assessment of premalignant lesions and as a tumor progression biomarker for advanced grades of OSCC.

Keywords: Speckled Leukoplakia, Oral Squamous Cell Carcinoma, Podoplanin, Biomarker, Immunohistochemistry, Epithelial Dysplasia

Introduction

In 2005, WHO defined leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer." [1,2] Leukoplakia (OPL) is most frequent potentially malignant disorder in the oral cavity with prevalence from 0.59% to 0.65% in India and 3% worldwide. [3,4,5,6] Leukoplakia shows annual malignant transformation rate of 0.1 to 18%. [3,7,8] Speckled leukoplakia has greater risk of malignant transformation then patients of homogenous leukoplakia [9,10,11] There was a dose-response relationship between leukoplakia and the frequency and duration of tobacco use. [12,13] Smokers have a significantly high prevalence of leukoplakia [3,14]

Histopathologically, leukoplakia is termed as Epithelial Dysplasia. Histolopathologic assessment of epithelial dysplasia is the gold standard for determining the malignant transformation risk of leukoplakia; however the accuracy of the histopathologic assessment of epithelial dysplasia depends on the quality of the tissue, the site at which a biopsy is taken, and with interobserver and intra-observer variability. Therefore, the identification of biologic and molecular factors needed to identify high-risk lesions. [15,16]

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck region with an annual incidence of more than 5,00,000 cases worldwide. [17,18,19,20] Despite advances in diagnostic and therapeutic modalities, the prognosis of OSCC still remains poor; thus, it is essential to identify novel biomarkers of tumor progression that will facilitate treatment selection. Immunohistochemistry (IHC) is an extraordinarily powerful tool in which there is utilization of monoclonal and polyclonal antibodies for the detection and tissue distribution of specific antigens in tissue sections. [21]

Podoplanin is a small mucin-like transmembrane protein and is a lymphatic endothelial marker, is highly expressed into oral cancer and some hyperplastic and dysplastic oral premalignant lesion and has been highest in aggressive tumors with higher invasive and metastatic potential. [15,22] Podoplanin was first used to identify Lymphatic vessels, but it was later shown that podoplanin is a useful marker of some malignant tumors. Hence, the purpose of this study is to evaluate the expression of podoplanin as a biomarker for cancer risk assessment in speckled leukoplakia and correlation of podoplanin with grades of OSCC.

Materials and Methods

Study material: In the present study, total 30 patients were included. From them, 15 patients were of speckled leukoplakia in which 12 were males and 3 were females with age range of 30-60 year while 15 patients were of Oral squamous cell carcinoma in which 12 were males and 3 were females with age range of 30-60 year. Detailed clinical history of each patient was recorded and incisional biopsy was collected for routine histopathological examination and for immunohistochemical (IHC) study for podoplanin expression.

- **Inclusion criteria:** Clinically diagnosed and histopathologically confirmed cases of Speckled leukoplakia and Oral squamous cell carcinoma.
- **Exclusion Criteria:** Homogeneous leukoplakia is not to be included in study.

Ethical clearance for the study was obtained from the Institutional Ethical Committee and Review Board. The patients were explained regarding the study procedure and written consent was obtained.

Histopathological study: Incisional biopsy of 2-3cm was performed from lesional patients under 2% lignocaine and excised tissues were used for the histopathologic examination. The biopsy tissue was fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin wax, 4-5 µm thick sections were cut and slides were prepared for routine Haematoxylin and Eosin stain. For IHC study 3-4 µm sections were prepared. H&E stained slides were used for the confirmation of histopathological diagnosis of the lesion and to identify the degree of dysplasia in oral leukoplakia and histopathological grades of oral squamous cell carcinoma.

Histopathological analysis

- Epithelial dysplasia was diagnosed and graded as Mild dysplasia, Moderate dysplasia, Severe dysplasia. [1,23]
- Oral squamous cell carcinoma was histopathologially diagnosed and graded according to WHO classification of Head and Neck Tumors based on Broders criteria as well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma. [24,25,26,27]

Immunohistochemical study: 3-4 µm sections were immunohistochemically stained with antibody against Podoplanin using the streptavidin-biotin technique. All 30 lesional tissue specimen were stained along with appendix tissue section as positive control. Then the slides were evaluated to determine the expression and staining intensity of podoplanin in the epithelium of speckled leukoplakia and OSCC.

Immunohistochemical analysis: In speckled leukoplakia, scoring of immunostaining was made using a scoring system described by **Kawaguchi et al (2008)** as follows:[28]

0—if no expression was observed in any part of the epithelium.

1—if expression was restricted to the basal layer of the epithelium.

2—if expression was observed in the basal and suprabasal layers at one area.

3—if the suprabasal layer expression was observed at two or three areas.

4—if the suprabasal layer expression was observed at more than three areas.

Score was calculated in 10 high power fields in each slide and mean was calculated per slide.

Calculation of cancer risk **according to Kawaguchi et al (2008)** as follows:[28]

Score 0-1: low risk or negative expression.

Score 2 or more: high risk or positive expression.

In OSCC, podoplanin expression was scored as described by **Yuan P et al 2006** and consider by **Patil A et al (2015)** and **Rodrigo JP et al, 2010 according to quantity of positive tumor cells** on a scale of 0 to 5 as follows: [18,29,30]

0: negative.

1: less than 10%.

2: more than 10% and less than 30%.

3: more than 30% and less than 50%.

4: more than 50% and less than 80%.

5: more than 80% positive staining.

Based on the staining intensity, positive specimens were classified into 4 categories:

3 = strong—dark brown staining of cells.

2 = moderate—staining between 2 extremes (dark brown and weak staining).

1 = weak—faint staining.

0 = negative—no staining.

German Immunoreactive Score (IRS) was calculated by multiplying quantity score and staining intensity scores. Scores could range from 0 to 15:

0 to 6 = weak reactivity and 7 or higher = high reactivity

Score was calculated in 10 high power fields in each slide and mean was calculated per slide.

Statistical analysis: All the data obtained were statistically analysed by using CHISQUARE TEST and compared for the expression of Podoplanin in the epithelium of speckled leukoplakia and OSCC.

Results

Out of total 30 patients, all the 15 patients of speckled leukoplakia were in age range of 30-60 years with the mean age of 44.1 years whereas 15 patients of OSCC were in age range of 30-60 years with the mean age of 45.06 years. Amongst 15 patients of speckled leukoplakia, 12(80%) patients were men and 3(20%) patients were women. Amongst 15 patients of OSCC, 12(80%) patients were men and 3(20%) patients were women.

The findings of podoplanin expression in speckled leukoplakia patients showed that all 6(100%) patients with severe dysplasia had high risk with score 3 & score 4 while, all 6(100%) patients with moderate dysplasia had high risk with score 2 & score 3 but only 1(100%) patients with mild dysplasia had low risk with score 1. Whereas 2 patients of speckled leukoplakia had no dysplasia. Podoplanin expression in patients with speckled leukoplakia having mild dysplasia showed low risk while in moderate and severe dysplasia showed high risk which is statistically significant **(p value=0.02)** as shown in **Table 1.**

Out of 15 patients with OSCC, 8(53.33%) patients had well differentiated squamous cell carcinoma, from them 1(12.50%) patient had score 0 and 2(25%) patients had score 2 while 5(62.50%) patients had score 6 of podoplanin expression means weak reactivity. 7(46.67%) patients had moderately differentiated squamous cell carcinoma, from them 3(42.86%) patients had score 6 of podoplanin expression means weak reactivity, 4(57.14%) patients had score 7 and more of podoplanin expression means high reactivity. Present findings showed that more than 50% patients with moderately differentiated squamous cell carcinoma showed high reactivity of podoplanin expression while, 100% patients with well differentiated squamous cell carcinoma showed weak reactivity of podoplanin expression which was statistically significant (p value0.013) as shown in **Table 2**.

Present findings showed that Patients with speckled leukoplakia, all 100% patients with moderate dysplasia and severe dysplasia had high risk but patients with mild dysplasia had low risk. Patient with OSCC, more than 50% patients with moderately differentiated squamous cell carcinoma showed high reactivity of podoplanin expression. While, 100% patients with well differentiated squamous cell carcinoma showed weak reactivity of podoplanin expression.

Discussion

OSCC is frequently preceded by potentially malignant disorders.[15] Because of the poor prognosis of OSCC, identification of reliable markers that indicate a high risk of malignant transformation in potentially malignant lesions is important.[31] Recent studies have identified podoplanin as a potential marker for malignant progression from oral leukoplakia to invasive carcinoma.[28] The present study was carried out to assess the potential associations between podoplanin expression and malignant transformation including the grade of dysplasia in oral leukoplakia and compare them with OSCC. In most of the patients included in the study, lesions were predominant in 5th decades in both the sexes with men predominancy.

Induction of podoplanin expression results in multiple adjustments of intracellular signalling pathways leading to the modulation of Rho family GTPase activities, the phosphorylation of ERM (Ezrin, Radixin, Moesin) proteins, rearrangement of the actin cytoskeleton and, finally, enhances cell migration and invasion. [34-37]

Podoplanin expression induces complex changes of the tumor microenvironment which collectively promote tumor cell motility, invasion, and metastasis.[38] Podoplanin is a recent marker whose relationship with various tumor invasions raises the possibility to employ podoplanin as a biomarker for diagnosis and prognosis. It has been noted that the upward clonal expression of podoplanin beyond the basal layer of the epithelium in leukoplakia cases carry a significantly higher risk of cancer development than in patients with negative expression. The exact role played by podoplanin in tumorigenesis is not fully elucidated, the podoplanin might favour tumor invasion through cytoskeleton reorganization of tumor cells not only by collective cell migration, but also by single cell migration following the loss of E-cadherin and thus play a role in tumor development.[17,36] Podoplanin expression is induced very early in the OSCC transformation process, and can be used to identify premalignant lesions that are bound to develop into oral cancer and indicate that over 46% of lesions with notable podoplanin expression progressed to OSCC. In contrast, less than 14% of lesions without notable podoplanin expression progressed to OSCC.[39]

In present study, 80% patients with moderate and severe dysplasia showed increased podoplanin expression which indicate high risk of transformation into OSCC while 7% patients with mild dysplasia had low risk of transformation into OSCC which was statistically significant (P=0.002). It indicates that podoplanin expression increases with severity of dysplasia which is also noted by Raica M, Kreppel M, Aishwarya A, Suzuki inoue K, Parhar S.[22,31,41,42,43] Podoplanin expression was significantly associated with malignant transformation of the leukoplakia.[31] Podoplanin expression increases the probability of OSCC formation from histologically benign lesions by over 3-fold and indicate that over 80% of oral leukoplakias that express high levels of podoplanin convert to oral cancer.[39,44] **Kawaguchi et al, 2008** reported that the frequencies of podoplanin expression increase with increased severity of dysplasia, particularly from mild dysplasia to moderate or severe dysplasia. They noted 32% patients of OPL lesions with only hyperplasia were podoplanin-positive, 41% patients of lesions with mild dysplasia, 70% patients of lesions with moderate dysplasia, and 100% patients with severe dysplasia/carcinoma in situ exhibited positive podoplanin. In their studies, they showed that podoplanin is expressed in oral dysplastic and hyperplastic lesions with an increased risk of cancer development.[28]

In epithelial dysplasia, high podoplanin expression has been associated with an increased risk of progression to invasive cancer, suggesting that podoplanin could serve as a powerful biomarker to predict the risk for oral cancer development in patients with potentially malignant disorders. Podoplanin is highly expressed in some hyperplastic and dysplastic lesions adjacent to the primary tumors which indicates that the overexpression occurs early in head and neck tumorigenesis.[18,19] Whereas a study by **De vicente et al, 2013** noted that in potentially malignant disorders, there are molecular genetic traits in common with OSCC even in the absence of histologically defined dysplasia and suggested that podoplanin may act as a mediator of tumor cell invasion and metastasis.[15] **Kawaguchi et al, 2008** also noted that 37% of oral leukoplakia (OPL) lesions exhibit expression patterns similar to those found in OSCC. The expression patterns

correlate with dysplasia of the oral leukoplakia lesions in a grade-dependent manner and a higher rate of oral cancer development which suggests a role of podoplanin in oral cancer initiation and progression.[28] Podoplanin expression correlate with the clinicopathological features of the OSCC patients and there was a statistically significant correlation between high podoplanin expression and the presence of lymph node metastasis and poor histological grade.[35]

In Present study, 100% patients with well differentiated squamous cell carcinoma showed weak reactivity of podoplanin expression while in 58% patients with moderately differentiated squamous cell carcinoma had high reactivity of podoplanin expression which was statistically significant (p=0.013) which means that podoplanin expression increases with increasing grades of OSCC. This finding is somewhat correlated with finding of **Kim et al, 2015** and **Yuan et al, 2006** who noted 46% patients with moderately differentiated squamous cell carcinoma had low reactivity and 54% patients with moderately differentiated squamous cell carcinoma had high reactivity.[18,35] In contrast to present findings, **Kim et al, 2015** and **Prasad et al, 2015** noted 69% patients and 30% patients of well differentiated squamous cell carcinoma had weak reactivity and 31% patients and 11% patients with well differentiated squamous cell carcinoma had high reactivity respectively.[35,45]

Parhar et al, 2015 noted that the transition of severe dysplasia to well differentiated SCC, the difference between the mean of the epithelial score came to be statistically highly significant ($P < 0.001$). Thus, suggesting that podoplanin may help as a marker in diagnosing the early changes occurring in dysplastic lesions to OSCC. Podoplanin may play some role in the regulation of differentiation, growth, and tumor progression of OSCC. [15,29,36,43,46,47]

Thus, it recommend that Evaluation of podoplanin along with histopathological evaluation can also provide additional information like malignant transformation which cannot be detected merely by clinical and histopathological analysis. Podoplanin expression in premalignant lesion increases with increasing grades of dysplasia which suggest high risk of transformation into OSCC. Thus, Podoplanin can be used as a biomarker for early oral tumorigenesis and for malignant transformation risk assessment of premalignant lesions and as a tumor progression biomarker for advanced grades of OSCC. Podoplanin could serve as a target for more effective treatments to improve outcomes in patient with OSCC population.

Conclusion

Using Podoplanin as a biomarker one can decide progression of tumor, malignant transformation, invasion and treatment accordingly and it helps to evaluate the progress of treatment. So it is helpful in prevention of transformation of epithelial dysplasia into OSCC and early diagnosis of epithelial dysplasia can result in early treatment and prevent progression to invasive carcinoma. Podoplanin can also be used as a prognosis marker to evaluate the treatment of OSCC.

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Legend Tables and Figures

Table 1: Comparison of Podoplanin expression according to Grades of dysplasia in Speckled leukoplakia

Lesion	Total	Grades of Dysplasia No.(%)	Score of Podoplanin Expression				
			Low risk		High Risk		
			Score 0	Score 1	Score 2	Score 3	Score 4
Speckled leukoplakia	15	No dysplasia 2(13.33%)	2(100%)	-	-	-	-
		Mild1 (6.67%)	-	1(100%)	-	-	-
		Moderate 6(40%)	-	-	2(33.33%)	4(66.67%)	-
		Severe 6(40%)	-	-	-	1(16.67%)	5(83.33%)
Total	15	15(100%)	2(13.33%)	1(6.67%)	2(13.34%)	5(33.33%)	5(33.33%)

Low Risk : Score 0-1, High Risk : Score 2 or more

Chi-Square Tests			
	Value	df	P VALUE
Pearson Chi-Square	15	3	0.002

Table 2: Comparison of Podoplanin expression according to Histopathological Grading of oral squamous cell carcinoma

Lesion	Total	Histopathological Grading		Podoplanin Expression							
				Weak Reactivity							High Reactivity
				Score 0	Score 1	Score 2	Score 3	Score 4	Score 5	Score 6	Score 7 Or More
OSCC	15	Well-Differentiated OSCC	8(53.33%)	1(12.50%)	-	2(25%)	-	-	-	5(62.50%)	-
		Moderately Differentiated OSCC	7(46.67%)	-	-	-	-	-	-	3(42.86%)	4(57.14%)
Total			15	1(6.67%)	-	2(13.33%)	-	-	-	8(53.33%)	4(26.67%)

Chi-Square Tests			
	Value	Df	P VALUE
Pearson Chi-Square	6.234	1	0.013

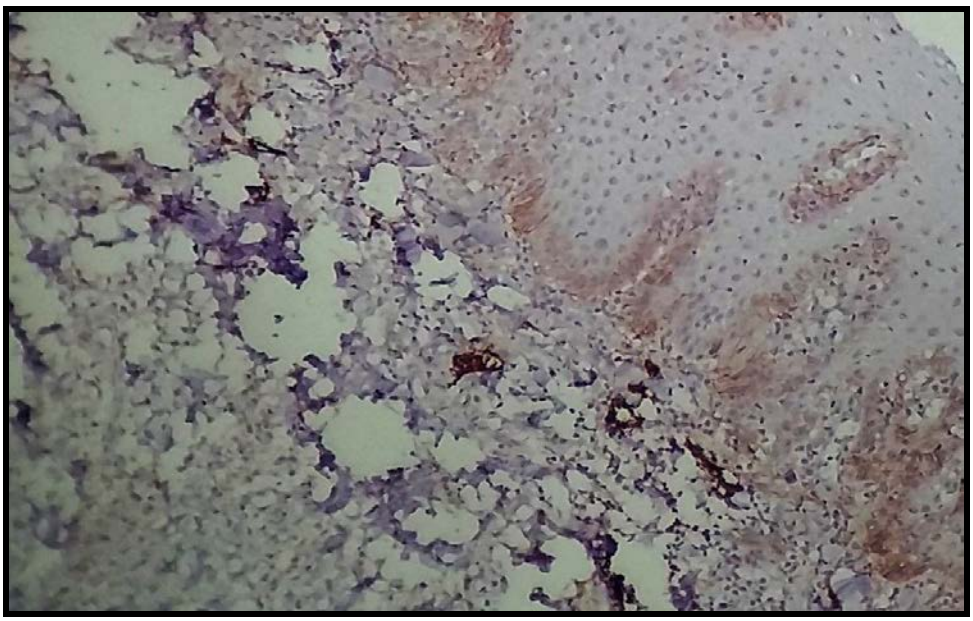

Figure 1: Histology of speckled leukoplakia with mild epithelial dysplasia (IHC stain, Magnification 10X)

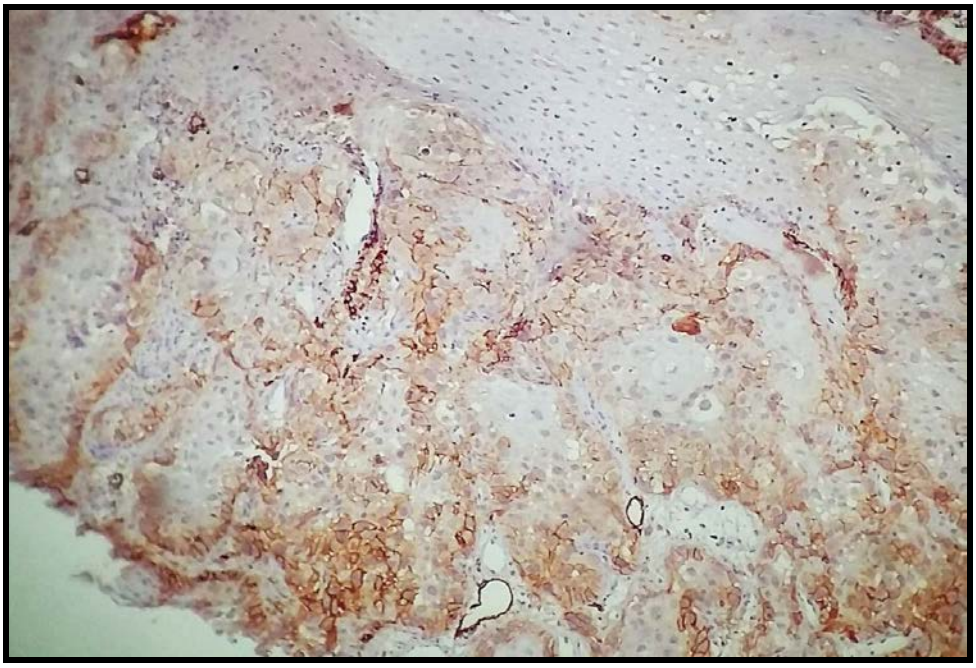

Figure 2: Histology of speckled leukoplakia with moderate epithelial dysplasia (IHC stain, Magnification 10X)

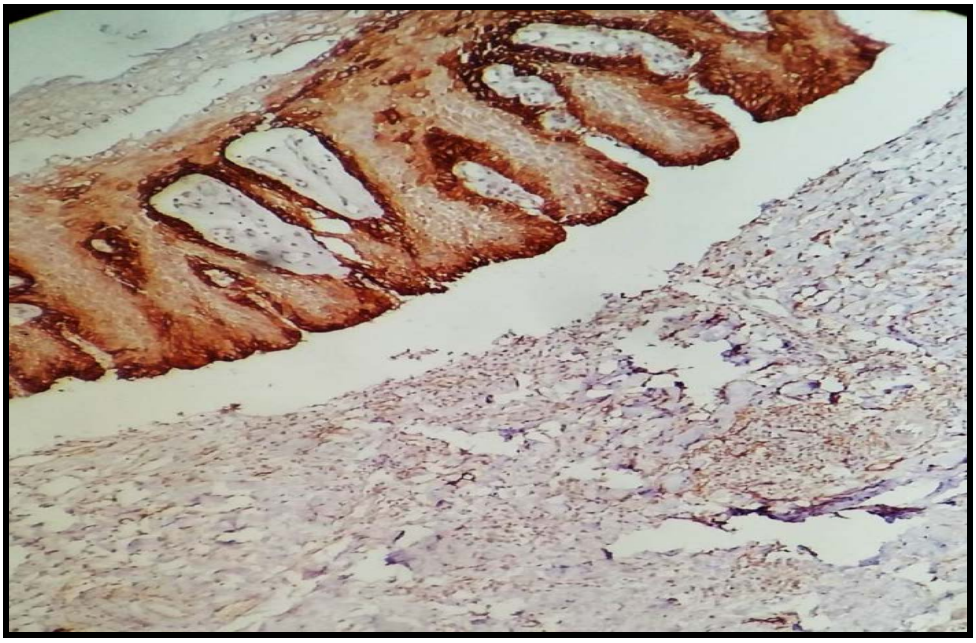

Figure 3: Histology of speckled leukoplakia with severe epithelial dysplasia (IHC stain, Magnification 40X)

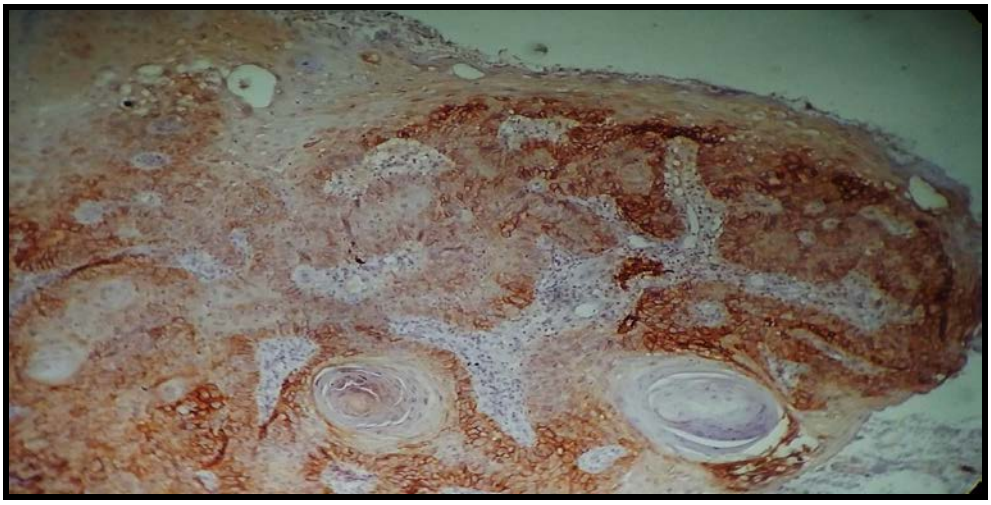

Figure 4: Histology of well-differentiated squamous cell carcinoma (IHC stain, Magnification 10X)

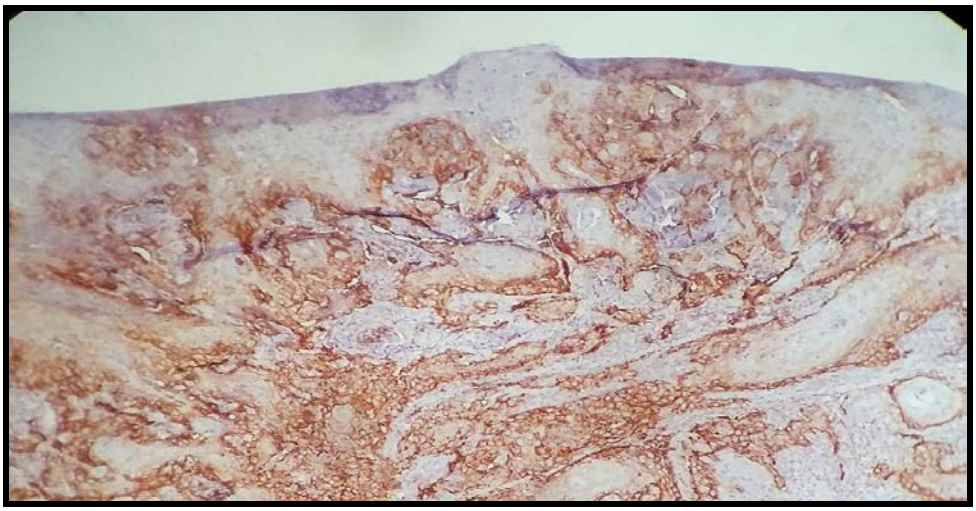

Figure 5: Histology of moderately differentiated squamous cell carcinoma (IHC stain, Magnification 10X)