Clinicopathological Correlation of Cd44 Expression and Carcinoma Associated Fibroblasts at the Invasive Tumour Front of Oral Squamous Cell Carcinoma- A Preliminary Study

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ABSTRACT

BACKGROUND AND AIM: Oral squamous cell carcinoma is one of the most prevalent malignant cancers in India. Invasive tumour front of OSCC shows lower degree of differentiation and higher grade of cellular dissociation when compared to other parts of tumour. Integral prognostic information about the invasiveness of tumour and its metastatic capacity can be deduced from ITF. This study is aimed to evaluate the expression of CD44 and Cancer Associated Fibroblasts at the Invasive Tumour Front of Oral Squamous Cell Carcinoma and to analyze the association between histopathological parameters and the expression of CD44 and CAF. This study also analyses the correlation between the expression of CD44 and the amount of CAF.

MATERIALS AND METHODS: 20 samples of histopathologically confirmed cases of OSCC were chosen and subjected to immunohistochemical Analysis using two antibodies Alpha smooth muscle actin (SMA) and CD44. The CD44 expression and CAFs were analyzed and correlated with the histopathological prognostic factors by using univariate and multivariate analysis using IBM SPSS Statistics 23 software.

RESULTS: Higher expression of CD44 and presence of more CAFs at the invasive front was associated with an increased depth of Invasion >5mm. This was found to be statistically significant in univariate analysis with p value=0.000 for CD44 expression and p value= 0.017 for CAF distribution respectively. High expression of CD44 was observed with the presence of more CAF at the invasive tumour front of OSCC cases selected (Chi square test-p value=0.025). 50% and 66.7%

of the lymph node infiltrated cases showed high distribution of CAF and high expression of CD44 respectively.

CONCLUSION: In the present study, we found that both high expression of CD44 and high distribution of CAF were associated with increased depth of invasion in OSCC, which is a very important prognostic factor. These factors can be used as markers for predicting invasiveness and can be used as targets in molecular cancer therapy.

KEYWORDS: CD44; Carcinoma Associated Fibroblast; Oral Squamous Cell Carcinoma; Invasive Tumour Front; Correlation

INTRODUCTION

Oral cancer is the 12th most common cancer and accounts, 2-4% of all cancer cases in the world (1). India has the highest number of oral cancer cases (approximately 20%) in the world(1). 90% of all the oral cancer cases fall under Oral Squamous Cell Carcinoma (OSCC) which is the most common malignancy of the oral cavity in India(1).

OSCC is a malignant neoplasm derived from the stratified squamous epithelium of the oral mucosa. Its etiopathogenesis is multifactorial, associated with cigarette smoke, alcohol, snuff and tobacco usage, as well as the papilloma virus (2).OSSC is characterized by the altered proliferation of dysplastic squamous cells on the surface of the epithelial layer, which subsequently degrades the basement membrane resulting in local destruction and distant invasion. Cancer cells at the progressing front of a tumor behave aggressively compared to cancer cells in the superficial or central regions of the main tumor mass(3). This most progressed, three to six tumor cell layers or detached tumor cell groups at the advancing edge of the OSCCs is termed as invasive tumor front (ITF) (4).ITF shows lower degree of differentiation and higher grade of cellular dissociation when compared to other parts of the tumor. Integral prognostic information about the invasiveness of the tumor and metastatic capacity can be deduced from ITF where processes such as gain and loss of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation, and initiation of angiogenesis occurs (4).

CD44 is a non-kinase, type 1, single span transmembrane glycoprotein also referred to as P-glycoprotein 1(5). It is ubiquitously expressed throughout the body and has a molecular weight of 85–200 kDa (6).It is encoded by a single gene containing 19 exons on chromosome locus 11p13 (7). The CD44 molecule has standard and variable isoforms(5). Standard isoform is represented as CD44s and variable isoform as CD44v (5,8). The functional role of different isoforms in cancer is complicated and not fully understood, butCD44s has been shown to promote the epithelial mesenchymal transition (EMT), CD44v act as a ligand for E-selectin during tumor metastasis and

CD44v expression is stimulated by hypoxia and hypoxia inducible factor-1 α (8). Considering all these functions CD44 has been described as a CSC marker in head and neck squamous cell carcinoma (HNSCC)(8). There are controversial results regarding CD44 expression level in OSCC and lymph node metastasis. Studies by Fonseca et al,Lindquist et al found a positive relationship between high CD44 expression and lymph-node metastasis, and by Mostaan et al, Rodrigo et al found correlation between low CD44 expression and capability for metastasis (9).

Cancer-associated fibroblasts (CAFs) are the most abundant and critical cellular component of tumor microenvironment in a variety of solid tumors(10). CAFs play a role in tumor development via cell-cell interaction or cross-talk with tumor cells by secreting growth factors, cytokines, and exosomes (11). CAFs produce ultrastructure of alpha-smooth muscle actin (αSMA) and demonstrate the characteristics of myofibroblast-differentiation (10).The role of CAF as promoter of tumor initiation, progression, epithelial to mesenchymal transition, stemness, tumor invasion, angiogenesis, and metastasis are well established (12). Studies have demonstrated that oral cancer patients whose tumors had lower levels of stromal-αSMA expression had significantly longer disease free and good prognosis(13).

This study aimed to evaluate the expression of CD44 and Cancer Associated Fibroblasts at the Invasive Tumor Front of Oral Squamous Cell Carcinoma and also aimed to analyze the association between histopathological parameters and the expression of CD44 and CAF. The present study also analysed the correlation between the expression of CD44 and the amount of CAF.

MATERIALS AND METHODS

This study was conducted after getting approval from the institutional ethics committee of Saveetha dental college and hospital Chennai during December 2019.

Sample Selection: This study included 20 cases of histopathologically confirmed cases ofOSCC reported to the department of oral and maxillofacial pathology during 2018-2019. The patients had not received prior radiation or chemotherapy. Formalin fixed paraffin embedded blocks of all the cases were retrieved from the archives of the Department of Oral and Maxillofacial Pathology.

Microscopic assessment of Histopathologic parameters:

The criteria used for evaluating Pattern of Invasion in this study was Brandwein – Gensler's risk scoring system. In this study, pushing border was given a score of 1 followed by finger-like growth, large separated island, small tumour island and tumour satellite graded as 2, 3, 4 and 5 respectively. Histopathological depth of invasion in this study was categorised as low risk and high risk with depth of invasion less than 5mm as low risk and greater than 5mm as high risk respectively. Tumour budding in this study was classified into low and high. Clusters of less than 5 buds were

categorised as low and clusters greater than 5 were categorised as low. Inflammatory response was graded into Pattern 3 (slight), Pattern 2 (moderate) and Pattern 1 (severe).

Immunohistochemistry (IHC)

For immunohistochemical study, 3 μm sections were cut from formalin fixed paraffin embedded blocks, mounted on gelatin coated slides. The sections were deparaffinized in xylene, dehydrated in alcohol and rinsed in distilled water. Antigen retrieval was performed using heat induced - epitope retrieval in citrate buffer (pH 6.0) for 10 min in a pressure cooker. Following which endogenous peroxidase was blocked for 5 min, protein block for 5 min. Sections were then incubated with Mouse Monoclonal Alpha Smooth Muscle (ASM-1)and CD 44 (156 3C-11). Detection was performed using Supersensitive ™ Polymer HRP IHC Detection System (DAKO Agilent Denmark). The sections were then counterstained with Mayer's hematoxylin. The slides were then dehydrated and mounted. Negative and positive controls were used in each run. Leiomyosarcoma sections were used as a positive control for alpha SMA and the blood vessels in the sections acted as internal control. Skin sections were used as a positive control for CD44. Negative controls for both CD44 and alpha SMA were achieved by performing the staining procedures with the omission of the primary antibody.

Immunohistochemical assessment:

Immunohistochemical assessment was carried out by 2 independent observers. The scoring was based on a combination of staining intensity and proportion of cells which are positively stained. The staining intensity was categorized as Nil, Weak, Moderate and Intense with scoring of 0, 1, 2 and 3 respectively. The proportion of cells stained were categorized as Negative, Focal and diffuse with scoring of 0, 1 and 2 respectively.

The combination of both proportion of the cells and staining intensity score were graded as Negative (0), Low(1,2), Moderate(3) and High (4,5). Based on this grading the expression of CD44 and CAF distribution were calculated. CD44 expression was also categorized based on location of expression as cytoplasmic and membranous.

Statistical analysis: IBM SPSS statistics 23 software was used for statistical analysis. Univariate analysis was used to analyse the association between the histopathological parameters and expression of CD44 and CAF distribution. In the univariate analysis and Chi-square test and Spearman correlation analysis $P < 0.05$ was considered statistically significant.

RESULTS

In the present study, 20 OSCC cases were analysed by Immunohistochemical staining to evaluate the expression of CD44 and amount of CAF at invasive tumour front. Out of the samples

selected, 85% were males and 15% were females. Gingivobuccal sulcus (50%) was found to be the most predominantly affected site. The demographic data and site are described in Table 1.

Histopathological evaluation of the cases revealed that 70% of the cases were WDSCC and 30% were MDSCC. Lymph node infiltration was observed in 30% of the cases and 10% of the cases showed perineural invasion. Extraxcapsular spread was present in 5% of the cases. Tumour budding was high in 35% of the cases and 45% of the cases showed moderate inflammatory response. Table 2 depicts the histopathological parameters of the selected cases.

The quantitative immunoexpression showed that CD44 was highly expressed (Figures 1 and 2) in 60 % of the cases followed by moderate, low (Figure 3) and negative(Figure 4) expressions in 10%,10% and 15% of the cases respectively (Table 3). Nonspecific immunoexpression of CD44 was seen in 5% of cases . The localisation of CD44 was assessed excluding the nonspecific and negatively stained cases. Cytoplasmic localisation was found in 18.8% and Membranous localisation was observed in 81.2% of cases. Among CD44 with cytoplasmic localisation, 66.7% of the cases showed lymph node infiltration, whereas only 30.8% of the cases with membranous localisation showed lymph node infiltration.This is represented in Table 4.

The distribution of CAF at the invasive tumour front was found to be high (Figure 5) in 50% of the cases followed by moderate in 35% and negative in 10% of cases (Table 3). 5% of the cases showed nonspecific immunostaining. The presence of CAF in the stroma adjacent to tumour island is represented in Figure 6.

Higher expression of CD44 and presence of more CAFs at the invasive front was associated with an increased depth of Invasion >5mm. This was found to be statistically significant in univariate analysis (Table 5 & 6)

Higher expression of CD 44 and increased CAFs at the invasive front was observed in cases with lymph node metastases. 50% of cases with high fibroblasts and 66.7% of cases with an increased CD44 expression had Lymph Node infiltration. However it was not found to be statistically significant with p values 0.607, 0.121 respectively. The correlation between the immunoexpression of CD44, presence of CAFs and the histological parameters are summarised in Table 5 and 6 respectively.

High expression of CD44 was associated with presence of more CAF at the invasive tumour front of OSCC cases selected. The present sample size was smaller to establish an association between CAF and CD44 expression, however Chi-square test was found to be statistically significant with a p value= 0.025.

DISCUSSION

CD44 is a transmembrane glycoprotein that regulates many functions like cell adhesion, migration and proliferation, angiogenesis, inflammation and tumour development (6). CD44 is widely distributed in normal adult and fetal tissues and plays a role in cell to cell and cell to matrix adherence. CD44 also plays critical roles in stemness regulation of cancer stem cells (CSCs) by helping in stem cell homing and migration (14). The present study was an attempt to analyse the expression of CD44 and CAF and their interplay at the invasive tumour front and correlate its levels with other histopathological prognosticators.

In the present study a high immunoexpression of CD44 was observed in 60% of the OSCC samples. There found to be a high expression of CD44 in 66.7% of the cases that showed lymph node metastasis. Similar observations were also reported by several researchers(15,16). Upregulation of CD44 expression can lead to loss of epithelial adhesion receptors like E-cadherin, occludin, claudins, and α-catenin as well as disassociation of β-catenin from adherens junctions, which can lead to invasion and metastasis. CD 44 promotes tumour progression by interacting with CD44 matrix metalloproteinase 9 (MMP9) secreted by fibroblast. This causes localisation of MMP 9 on the cell surface leading to tumour invasion(17). This could be one of the reasons for the increased expression of CD44 in lymph node infiltrated cases. We could also find that increased expression of CD44 was associated with Lymph Node infiltration and this was found to be statistically Significant.

Souichi Yanamoto et al in 2014 stated in their study that the expression of CD44v6 was significantly associated with regional lymph node infiltration, pattern of invasion, depth of invasion, perineural invasion and local recurrence (18). However, in the present study, higher expression of CD44 at the invasive front was only associated with the increased depth of Invasion >5mm. Similar results were reported by Morand et al in 2019, (18,19). The increased depth of invasion in cases with increased CD44 expression suggestive of EMT, increased extracellular matrix remodelling and CSC signaling enabling tumor to invade and to metastasize(20).

CD44 is considered as a surrogate for cancer stem cells. In the present study, 66.7% of cases with intense cytoplasmic localisation of CD44, suggestive of possible CSC phenotype. Clinically these lesions had a higher tumour grade, depth of invasion (up to 10mm) and lymph node metastases.

Fibroblasts in normal stroma have tumour-suppressing properties (12). When the normal fibroblasts are activated into myofibroblasts they lose their tumour-suppressing functions and under suitable conditions convert into tumour promoting CAFs (12). The expression of alpha-smooth muscle actin in CAF represents the myofibroblast differentiation (10). There were several studies

using experimental models that provided the direct evidence of the role of CAFs in initiation of cancer (21). The important role of CAF in dissemination of tumour by the induction of a motile and invasive phenotype in tumour cells and extracellular matrix remodelling were also studied. (10). CAF-mediated ECM remodelling provides a supportive microenvironment for tumour cell migration and dissemination (10). Therefore we also correlated the CAFs along with histopathological parameters at the ITF.

In the present study, 50% of the OSCC cases showed high distribution of CAF at the invasive tumour front and also 50% of the cases with lymph node infiltration showed high CAF distribution. Several studies have also reported poor prognosis for OSCC patients, where the stroma expressed myofibroblastic-αSMA-positive CAFs (22). In this study also the results were supporting the above observation. However, there was no statistically significant correlation found between the distribution of CAF and lymph node infiltration. This might be attributed to the smaller samples size of the present study.

There was a statistically significant association between the depth of invasion and increased distribution of CAF. This could be attributed to CAF induced remodelling of ECM, epithelial mesenchymal transition (EMT) and induction of motile phenotypes(12). The extracellular matrix factor, Hyaluronic acid (HA) produced by CAF, plays a significant role in the migratory interactions of CAFs and inducing motile phenotypes in tumor cells(23).

Some researchers have shown that in aggressive cases, the tumour cells can acquire mesenchymal characteristics and can develop autocrine production of HA to sustain their high motility rates (24). From these studies it was observed that CAF utilises HA to migrate towards tumor cells and their autocrine production of HA and the binding of the HA to the HA binding receptor on the tumour cell surface stimulates the migration of the tumor cell populations. The binding of HA to the tumour cells by the receptors or cell surface proteins such as CD44 and RHAMM(12). Some studies have also reported the circulating tumour cells obtained from cancer patients expressed the CD44 receptor for hyaluronic acid (25). These circulating tumour cells with CD44 receptor and having dual phenotype of epithelium and connective tissue may represent tumor cells that have undergone EMT or circulating cancer stem cells (CSCs). This could be one of the reasons for the statistically significant association between the increased distribution of CAF and high expression of CD44 ($p=0.025$). This interaction between CAF and CD44 receptors via the secreted extracellular matrix factors play a major role in tumour invasion and metastasis.

The present study was an attempt to study the CD44 expression and CAF at the invasive front. The study has some limitations.The sample size is limited as it was carried out as an initial phase pilot study to an in vitro molecular study in OSCC. However, more extensive in vitro studies need to be conducted to analyse the mechanism of interaction of variants of CD44 with CAF and

the role of CD44 variants in the tumour invasion and metastasis to exploit them as potential therapeutic targets.

CONCLUSION

In the present study, we found that both high expression of CD44 and high distribution of CAF were associated with increased depth of invasion in OSCC, which is a very important prognostic factor. These factors can be used as markers for predicting invasiveness and can be used as targets in molecular cancer therapy. However extensive studies need to be done further to evaluate the functions and extensive interactions of these two factors.

CONFLICTS OF INTEREST

There are no conflicts of interests.

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List of Tables

Table 1: Demographic and clinical data of the samples included in the study

Sample Size	**20**
Gender Male Female	 85% 15%
Site Of The Lesion Gingivobuccal Sulcus Lateral Border of tongue Buccal mucosa	 50% 30% 20%
Lymph Node infiltration	30%
Survival Rate	90%

Table 2: Histopathological characteristics of the cases included in the study

Histopathological features		**Percentage**
Grade	WDSCC	75%

	MDSCC	25%
Pattern of Invasion	Pushing Border	5%
	Large Separate islands, >15 cells per island	10%
	Small tumour islands<15 cells /island	85%
Tumour Budding	Low	35%
	High	65%
Perineural invasion	Present	10%
	Absent	90%
Extracapsular spread	Present	5%
	Absent	95%
Inflammatory infiltrate	Pattern 1	30%
	pattern 2	45%
	Pattern 3	25%

Table 3:CD 44 expression and distribution of stromal CAF at the invasive front of OSCC cases

GRADINGS	**CD44**	**Distribution CAF**
Negative	15%	10%
Low	10%	0%
Moderate	10%	35%
High	60%	50%
nonspecific	5%	5%

Table 4: localisation of CD44 and lymph node infiltration status

CD44 localisation	**Lymph node infiltration**	
	Present	**Absent**
cytoplasmic(18.8%)	66.7%	33.3%
membranous(81.2%)	30.8%	69.2%

Table 5: Univariate analysis of histopathological variables for CD44 expression

Histopathological Parameters		**CD44 expression**					**Univariate analysis**
		Negative	**Low**	**Moderate**	**High**	**Non specific**	**p value**
Pattern of invasion	Pushing border	0	0	0	1	0	0.912
	Large separate islands	0	0	1	1	0	
	Small separate islands	3	2	1	10	1	
Shape of tumour nest	Oval shaped islands	0	0	1	1	0	0.431
	Scattered	3	2	1	11	1	
Depth of invasion	<5 mm	1	0	1	4	0	*0.00
	≥5 mm	2	2	1	8	1	
Tumour	<5 cluster	1	2	2	5	1	0.272

budding	>5 cluster	2	0	0	7	0	
Extracapsular spread	Absent	3	2	2	11	1	0.966
	Present	0	0	0	1	0	
Inflammatory response	pattern 3	0	1	0	2	0	0.460
	Pattern 2	2	1	1	6	0	
	Pattern 1	1	0	1	4	1	
Perineural invasion	Absent	3	2	2	10	1	0.873
	present	0	0	0	2	0	
LN infiltration	Absent	3	0	2	8	1	0.124
	Present	0	2	0	4	0	

*p value <0.05 - statistically significant

Table 6: Univariate analysis of histopathological variables for CAF expression

Histopathological Parameters		**CAF Distribution**				**Univariate analysis**
		Negative	**Moderate**	**High**	**Non specific**	**p value**
Pattern of invasion	Pushing border	0	0	1	0	0.836
	Large separate islands	0	1	1	0	
	Small separate islands	1	6	8	2	
Shape of tumour	Oval shaped islands	0	0	2	0	0.585
	Scattered	1	7	8	2	

Depth of invasion	<5 mm	1	3	2	0	*0.017
	≥5 mm	0	4	8	2	
Tumour budding	<5 cluster	0	4	6	1	0.763
	>5 cluster	1	3	4	1	
Extracapsular spread	Absent	1	7	9	2	0.828
	Present	0	0	1	0	
Inflammatory response	pattern 3	0	2	1	0	0.774
	Pattern 2	1	3	5	1	
	Pattern 1	0	2	4	1	
Perineural invasion	Absent	1	6	9	2	0.873
	present	0	1	1	0	
LN infiltration	Absent	1	4	7	2	0.662
	Present	0	3	3	0	

*p value <0.05 - statistically significant

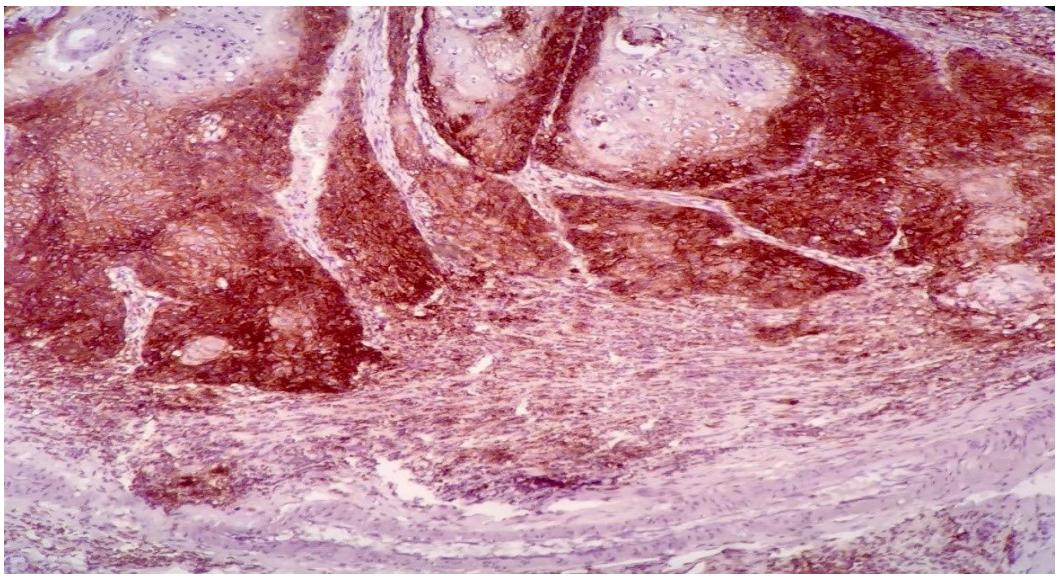

Fig 1: High CD44 staining at the ITF with cytoplasmic localization. Clinically the patient presented with lymph node metastasis (CD44, 10X magnification).

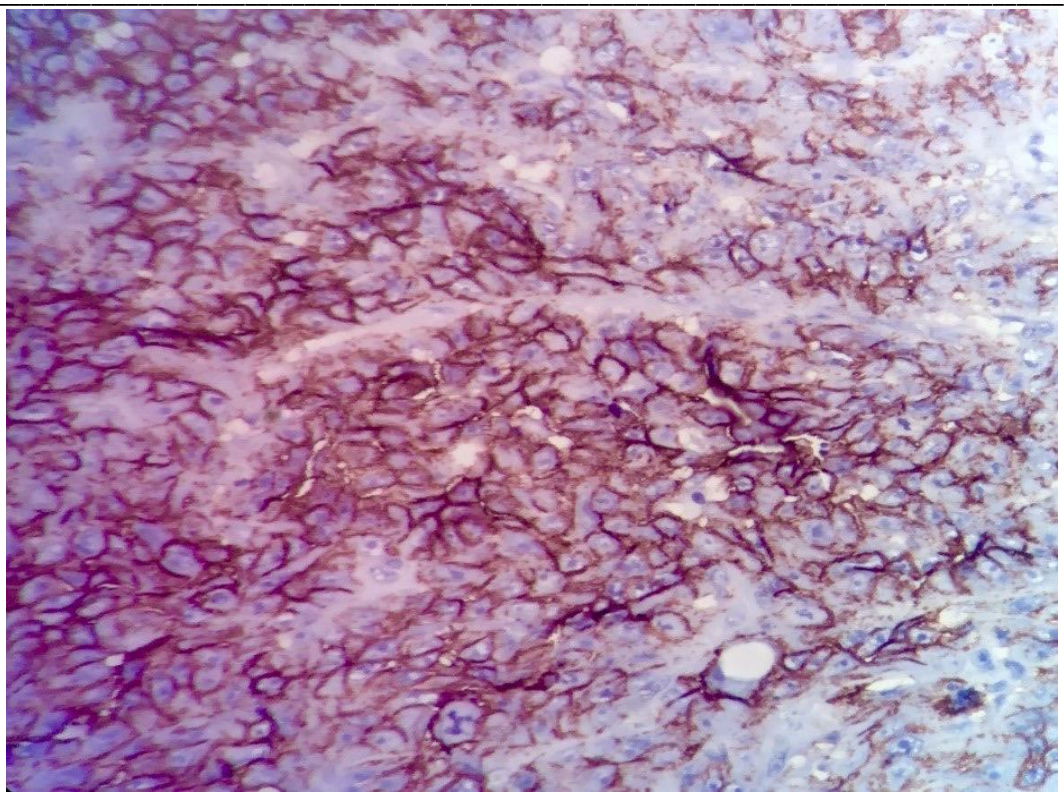

Figure 2: ITF with malignant cells showing membranous expression CD44 and loss of CD44 in some of the cells. Clinically the patient presented with lymph node metastasis (CD44, 40X magnification)

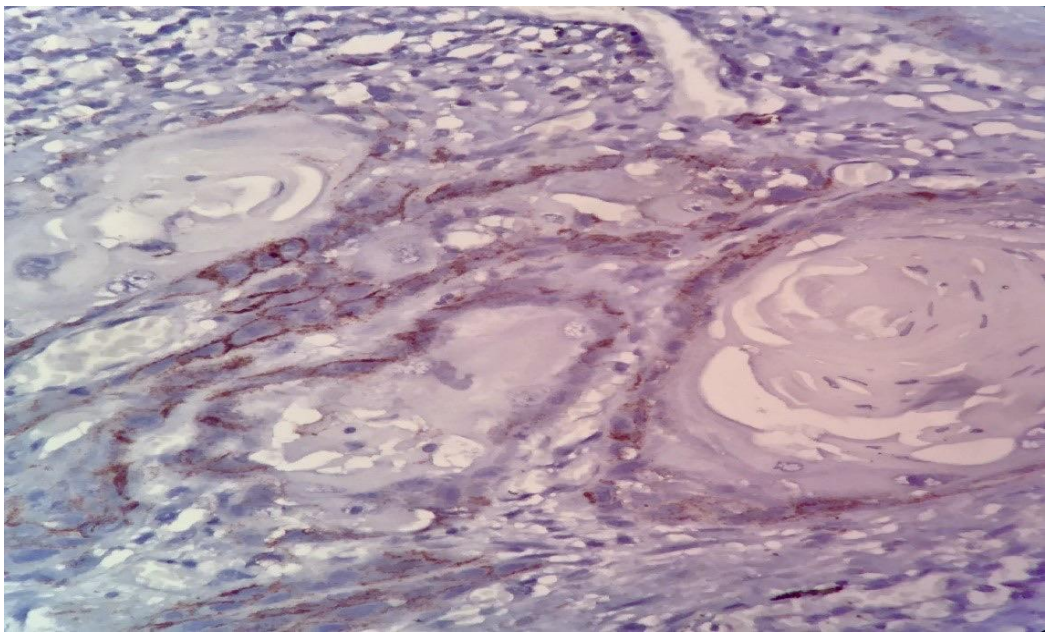

Figure 3: ITF with malignant cells showing low membranous expression CD44 and loss of CD44 in some of the cells. Clinically the patient presented with lymph node metastasis (CD44, 40X magnification)

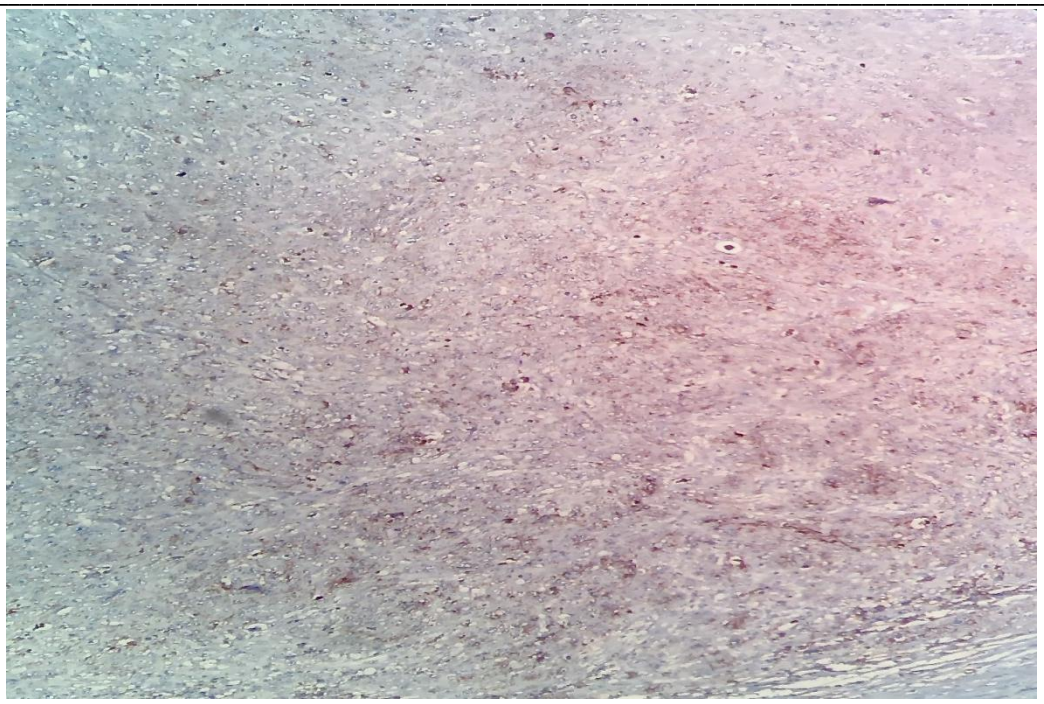

Figure 4: Photomicrograph showing negative expression of CD44. (CD44, 10X magnification)

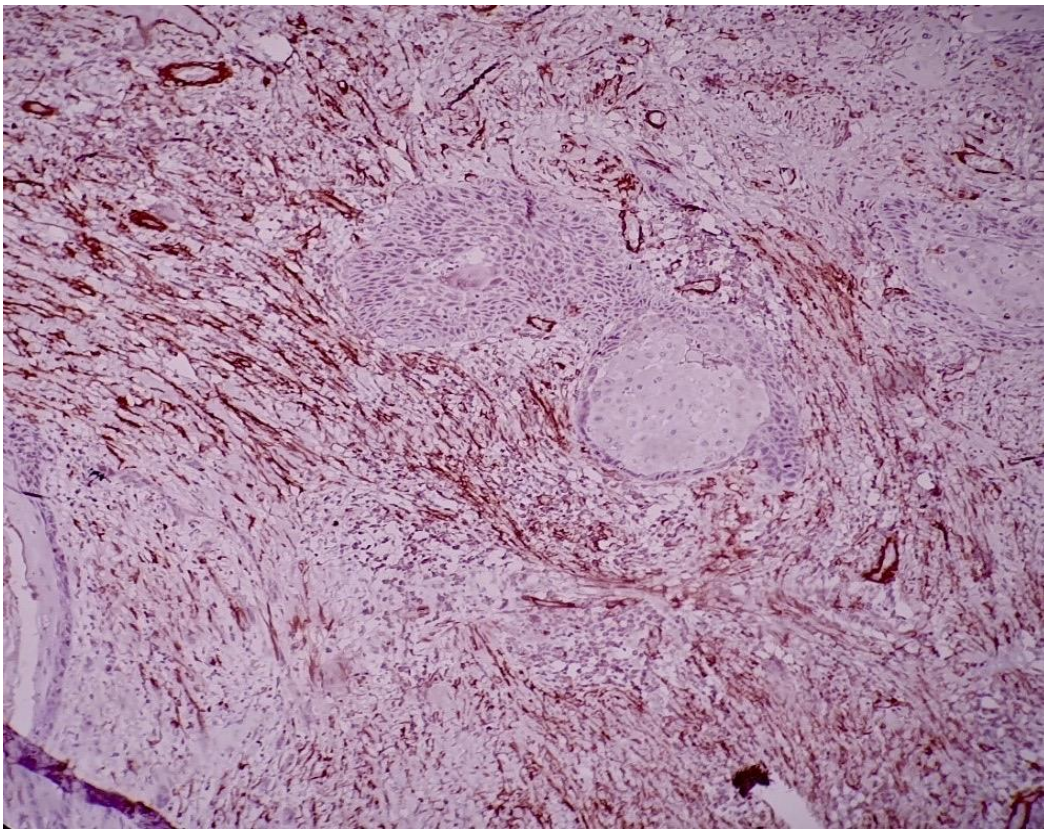

Figure 5: Photomicrograph showing high distribution of CAFs in the peritumoral area (Alpha SMA,10X magnification)

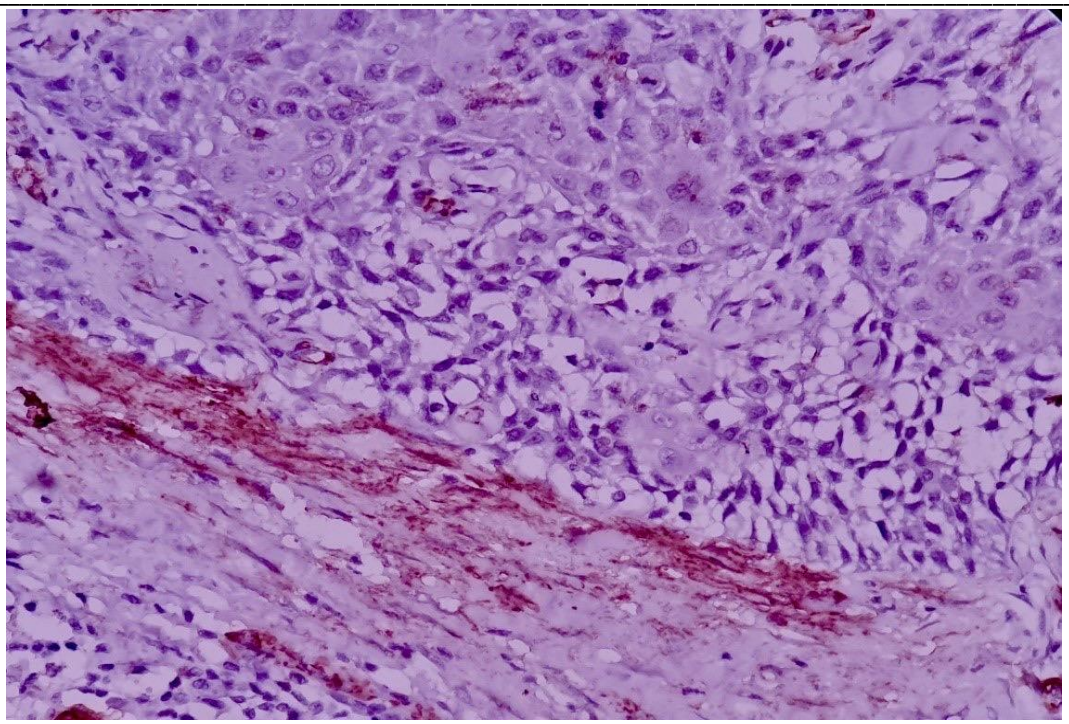

Figure 6: Photomicrograph showing CAFs in the adjacent stroma (Alpha SMA,40X magnification