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Role of Myofibroblast and Its Evaluation by Alpha Smooth Muscle Actin in Squamous Cell Carcinoma of Tongue and Buccal Mucosa In Relation To Lymph Node Metastasis

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Abstract

Background: Myofibroblasts are a relevant component in the stroma of oral cancer cells, however they are not present in all Oral Squamous Cell Carcinoma(OSCC) cases. These are involved in the contractile function during the invasion process of OSCC probably as a result of the modulation of the expression of growth factors, cytokines, extracellular matrix components and proteolytic enzyme.

Aims and Objectives: To determine the distribution of myofibroblast by their expression and distribution of α-SMA in Squamous cell carcinoma of tongue and buccal mucosa and to ascertain their possible role in the lymph node metastasis.

Materials And Methods: Twenty archival biopsy samples of diagnosed cases of squamous cell carcinoma of tongue and buccal mucosa were considered. Out of which ten biopsy samples with lymph node metastasis and ten without lymph node metastasis from both the sites were subjected to Immunohistochemical(IHC) staining with the help of marker α-SMA.

Results: In comparison to non-metastatic lesions, metastatic lesions showed an increase in the degree of α-SMA positive myofibroblasts. Statistically significant difference was present in our study among the metastatic and non-metastatic group at buccal mucosa but not on tongue.

Conclusion: Carcinomas with higher expression of myofibroblasts might be more aggressive and have poor prognosis hence are recommended for aggressive treatment and close follow up.

Introduction

Squamous cell carcinoma (SCC) of the oral cavity is one of the most common cancer worldwide. Approximately 94% of all oral malignancies are squamous cell carcinoma.[1] According to the SEER(U.S SURVELLEINCE OF EPIDEMIOLOGY END RESULTS) data tongue cancer accounts for 20% of all oral cavity and pharyngeal cancers.[1] SCC of the buccal mucosa accounts for approximately 10% of all cancers of the oral cavity reported in United States and 41% of all cancers of the oral cavity in India.[2] Metastatic squamous cell carcinoma (SCC) to regional lymph nodes (RLNs) plays a pivotal role in initial diagnosis, staging and management of oral SCC and is the single most important

prognostic indicator.[3] Approximately 30% of patients with intraoral SCC present with positive RLNs.[3]

Stromal myofibroblast (SMF) cells are known to emerge from stromal normal fibroblasts under the direct impact of cancer cell-derived cytokines.[4] Malignant epithelial cells are also a significant source of SMF if undergone Epithelial- mesenchymal transition (EMT).[5] SMF are aimed to further facilitate tumor to local and distant invasion as well as aid in the suppression of the host immune response due to the production of metalloproteinases which causes collagen breakdown.[4] Myofibroblasts possess greatly increased contractile ability.[4] It is also observed that SMF promote angiogenesis, and stimulate epithelial cell growth through the production of extra cellular matrix (ECM) and the secretion of growth factor and cytokines.[6] Myofibroblasts can be detected immunohistochemically by the presence of α-smooth muscle actin (α-SMA).SCC tongue is associated with the highest rate of metastasis as compared with other tumor sites in the oral cavity.[7]

However, only little work has been done in the evaluation of SMF in buccal mucosa (most common site for oral cavity SCC in India) and tongue in relation with the lymph node metastasis. Hence this study "Evaluation of myofibroblast by alpha smooth muscle actin in squamous cell carcinoma of tongue and buccal mucosa in relation to lymph node metastasis" was planned to know the role of α-SMA expression in correlation with the lymph node metastasis.

Materials and Methods

Formalin-fixed paraffin-embedded sections of 20 squamous cell carcinoma from tongue and buccal mucosa, were obtained from the archives or biopsy samples that came to Department of Oral Pathology and Microbiology, A. B. Shetty Memorial Institute of Dental Sciences, Deralakatte, Mangalore. Total sample size was 40.The study design consisted of two groups,

Group I

20 Biopsy samples diagnosed as squamous cell carcinoma of tongue after the routine tissue processing and haematoxylin and eosin staining technique were considered.

I a.) 10 Biopsy samples with level I lymph node metastasis without the involvement of the other groups. (Figure. 2)

II b.) 10 Biopsy samples without lymph node metastasis. (Figure. 3)

Group II

20 Biopsy samples diagnosed as squamous cell carcinoma of buccal mucosa after the routine tissue processing and haematoxylin and eosin staining technique were considered.

II a.) 10 Biopsy samples with level I lymph node metastasis without the involvement of the other groups

II b.) 10 Biopsy samples without lymph node metastasis.

Diagnosis of squamous cell carcinoma of buccal mucosa and squamous cell carcinoma of tongue was done histopathologically by haematoxylin-eosin staining. Sections of 3µm thickness were prepared for each squamous cell carcinoma of tongue and squamous cell carcinoma of buccal mucosa from the chosen paraffin wax blocks. IHC was done by using Biogenex life sciences pvt ltd. antibody kit for alpha-SMA Selected slides were coded to avoid the bias during the assessment of α-SMA expression.

Evaluation of α-SMA staining in the tissue sections

Staining was visualized with bright field microscope. The expression of the cells was evaluated at 40X magnification and scored by two observers to eliminate inter-observer bias. Sections were semi-quantitatively evaluated for α-SMA expression. Three fields were

randomly chosen. α-SMA were checked in non-inflammatory and non-endothelial stromal spindle cells, wherein cytoplasmic and/or membranous staining was considered positive.(Figure. 5) The areas between and adjacent to the tumor islands and the connective zone immediately adjacent to the invasive tumor front were considered for counting.(Figure. 2.) Criteria for evaluating the α-SMA staining in the tissue sections were that documented by Allred et al. The intensity and number of cells with positive expression among 100 cells in each field were counted and an average was calculated. According to Allred et al, a sum score of 2-6 is considered low above which is considered high. (Figure. 1, 5, 6)

Statistical Analysis

The data was entered in microsoft excel worksheet and analysed using IBM SPSS Version 22. Descriptive statistics were presented as frequencies, percentage, mean and Standard Deviation(S.D).Chi-square test used to assess the association among study variables with α-SMA sum score, Independent sample 't' test was used to compare the mean α-SMA score between study variables.

Results and Observations

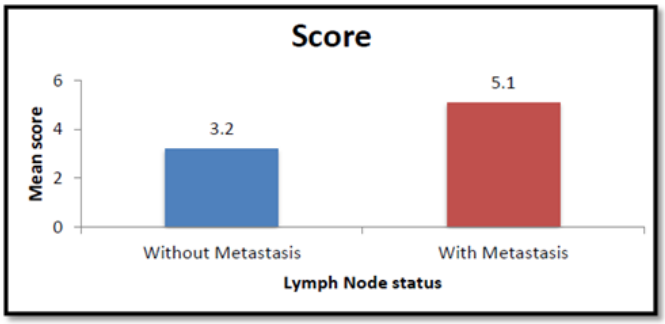
This study was done in order to assess the difference in expression and distribution of α-SMA in OSCC cases at two anatomical location i.e tongue and buccal mucosa with and without lymph node metastasis .The α-SMA sum score was calculated for each subject. Statistical analysis was carried out for any significance between squamous cell carcinoma of tongue and buccal mucosa with and without lymph node metastasis. The values of the probability value (p-value) was set at $p>0.05$ as non-significant (NS), $p<0.05$ as significant (S), $p<0.01$ as highly significant (HS), and $p<0.001$ as very highly significant (VHS). Positive staining was immunohistochemical brown cytoplasmic colour using α-SMA marker in myofibroblasts of the positive-stroma.

Lymph node status	Site	Score		Total	Fisher's exact test
		<6	7 and more		p-value
Without metastasis	Buccal Mucosa	10(50.0%)		10(50.0%)	-
	Tongue	10(50.0%)	0	10(50.0%)	
With metastasis	Buccal Mucosa	9(45.0%)	1(5.0%)	10(50.0%)	1.00(NS)
	Tongue	9(45.0%)	1(5.0%)	10(50.0%)	

Table 1: Frequency of IHC staining with α-SMA in OSCC with and without metastasis for both the sites.

Lymph node status	N	Mean	SD	Mean Difference (95% CI)	t	df	p-value
Without metastasis	20	3.20	1.57	-1.90(-3.020.77)	-3.42	38	0.001*
With metastasis	20	5.10	1.91				

Table 2: Comparison of mean α-SMA sum score of OSCC cases based on lymph node status



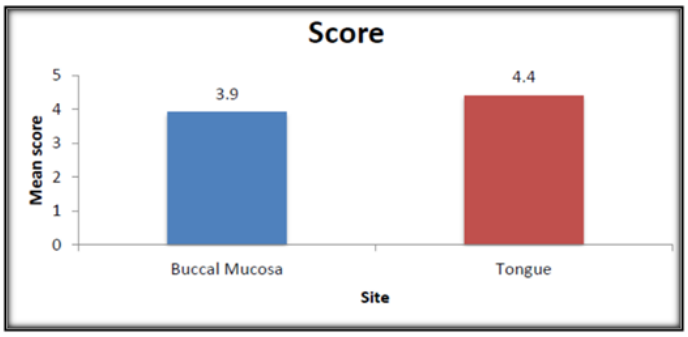
Graph 1: Comparison of mean α-SMA sum score of OSCC cases based on lymph node status

The difference in mean α-SMA sum score of metastasis and non-metastasis cases was statistically significant.(p=<0.001)(Table 2. and Graph 1.) The difference in mean α-SMA sum score among both the sites i.e buccal mucosa and tongue cases was not statistically significant(p=0.43)(Table 3. and Graph 2).

Site	N	Mean	SD	Mean Difference (95% CI)	t	df	p-value
Buccal mucosa	20	3.90	2.07	-0.50(-1.77, 0.77)	-0.79	38	0.43(NS)
Tongue	20	4.40	1.90				

Table 3: Comparison of mean α-SMA sum score of OSCC cases based on site.

Even though the difference in mean α-SMA sum score when compared among both the metastatic and non-metastatic in combined cases from both the anatomical sites i.e tongue and buccal mucosa were statistically significant however only buccal mucosa showed significant difference in α-SMA score in the metastatic cases compared with its non metastatic cases but this was not observed in the tongue cases. (buccal mucosa p-value= 0.01 & tongue p-value=0.06)(Table 4.)

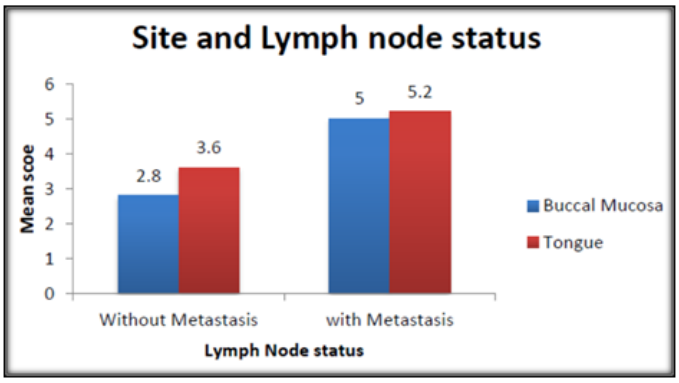


Graph 2: Comparison of mean α-SMA sum score of OSCC cases based on site

Out of total sample size(N=40), 5 cases (12.5%) did not show α-SMA myofibroblast expression completely whereas 35 cases (87.5%) cases did show positive expression. Out of all 35 cases which showed positivity for α-SMA myofibroblast, 17 cases were from buccal mucosa (48.6%) and 18 cases were from tongue(51.4%). Similarly out of all positive cases 19 cases (54.3%) showed metastasis to level I lymph node whereas 16 cases (45.7%) did not show metastasis.

Site	Lymph node status	N	Mean	SD	Mean Difference (95% CI)	t	Df	p-value
Buccal mucosa	Without metastasis	10	2.80	1.61	-2.20(-3.88, 0.52)	-2.75	18	0.01
	With metastasis	10	5.00	1.94				
Tongue	Without metastasis	10	3.60	1.50	-1.60(-3.25, 0.06)	-2.02	18	0.06
	With metastasis	10	5.20	1.98				

Table 4: Comparison of mean α-SMA sum score of OSCC cases with and without lymph node metastasis in both the anatomical sites.



Graph 3: Comparison of mean α-SMA sum score of OSCC cases with and without lymph node metastasis in both the anatomical sites.

Total number of cases which showed negative expression for α-SMA myofibroblast were 5(12.5%). Out of all the 3 cases (60%) were from buccal mucosa and 2 cases (40%) were from tongue. Metastasis to level I lymph node was seen only in 1 case (20%) and rest all 4 cases(80%) did not metastasize.

There are increasing amount of changes at the molecular level during the transformation of a dysplastic epithelium into squamous cell carcinoma. A defective response in both compartments i.e epithelium and lamina propria occur as a result of genetic and epigenetic factors which would be possible for carcinogenesis and tumour progression.[8]

Several studies have been known to show the role of carcinomatous changes in stroma leading to tumorigenesis, invasion and metastasis. The mechanism of stromal cells such as myofibroblasts having influence on neoplastic cells remains unclear. A revised nomenclature for malignant epithelial tumours, i.e'carcinoma' have even been suggested by Albini and Sporn. The alterations within the epithelium in SCC may not be the only factor responsible; in fact the different stromal factors may participate in its development via the interaction with the epithelial compartment. An important event which can be seen in the stroma of several invasive carcinomas is the trans-differentiation of fibroblasts to myofibroblasts.[8]

To study the expression of MF in the current study a total of 40 Formalin fixed, paraffin embedded tissue specimen of histopathologically diagnosed cases of oral squamous cell carcinoma were included. The clinical data was collected and tabulated and slides of each section stained immunohistochemically with α-SMA were scrutinized for frequency of expression and distribution of myofibroblasts. Similar to the observations made by Prabhu and Daftary, most cases of OSCC in the present study were in patients above 40 years, age ranged from 32- 72 years. Oral cancer like most other cancers, affect the individuals in the higher age group mostly above 40 years. International Agency for Research on Cancer (IARC) database quotes the peak incidence in India is in the 5th to 6th decades of life.[9] Out of 40 patients of OSCC 13 (32.5%) were females. Because of lesser sample size, this is not representative of the true incidence of OSCC in Indian women. Men are affected 2-3 times as often as women because of heavy indulgence of tobacco as per the IARC database reports in most of the countries. But due to heavy indulgence in tobacco chewing than in other registries, India has higher rates of females affected with oral cancer.[9]

α-SMA was the marker used for the detection of MFs in our study. The rationale behind selecting this marker was because of the fact that MFs are transdifferentiated from other cell types and they display prominent cytoplasmic actin microfilament which the parent cell (like fibroblast) often lack.[10] Other markers have been used in conjunction with α-SMA, for e.g TGF-β and EMA (Epithelial Membrane Antigen) by Vered et al. [11,12]. Desmin and Vimentin by Moghadam et al.[8] Vered et al used EMA together with α-SMA to study the transition of MF from epithelial cell and TGF-β to observe if the frequency of MF is affected by changes in expression of TGF-βl .This was done to prove the theory that epithelial cell undergo epithelial- mesenchymal transition to form MFs and the leading mediator of this transition of epithelial cells into mesenchymal cells was confirmed to be TGF β1 .Vimentin and desmin are mesenchymal markers also used to study the origin of MFs. Since our aim was only to study the expression and distribution of MFs, we used α-SMA which is the most reliable marker for identification of MF.

In the present study when α-SMA stained slides of OSCC (n=40) were evaluated for frequency of MF expression, we found that α-SMA positive myofibroblasts were expressed in 35 out of 40 cases of OSCC**.** These results were in agreement with those of Vered et al (2010), Chaudhary et al (2012), Vered et al (2009), and Moghadam et al(2009) who have reported 98%, 97%, 100% & 100% tumors respectively expressing MFs. Whereas Kellerman et al (2009), Safora Seifi et al. (2010), Kapse et al (2013) have reported the presence of α -SMA in 60% , 67%, 70% tumours only respectively. This variability in α-SMA positivity of myofibroblast in OSCC can be thought of because of varying expression of TGF-

β1 among them. Role of TGF-β1 is complex as it acts as tumor suppressor in premalignant phase whereas it becomes pro-oncogenic at later phases of cancer thereby facilitating in invasion and metastasis.[13] However, 9 out of 10 metastatic cases from both the sites showed positive immunostaining with α-SMA. Similarly, 9 out of 10 non-metastatic cases from tongue showed positive staining whereas 8 out of 10 buccal mucosa cases showed positivity. In the present study, MFs were found surrounding the tumor islands and cords in the stroma, and often in the deep invasive front of the tumors. Some tumors expressed only a few MFs in delicate rows surrounding and abutting the tumor islands, while others showed abundance of MFs in the stroma which were organized in syncytium. These differences might reflect the biological behaviour of the tumors. Vered et al have proposed that higher the number of MF more aggressive is the tumor with increased recurrence and poor survival rate.[11,12] This can be attributed to the fact that the MFs are 'factories, of a range of cytokines and growth factors like matrix metalloproteinases (MMPs), vascular endothelial growth factors (VEGF), fibroblast growth factor (FGF) etc. Through production of cytokines, chemokines and proteolytic enzymes MFs modulate the tumor stroma. They boost tumor angiogenesis, drive tumor invasion and metastasis thereby helping tumor progression. Increased frequency of MFs in the stroma has been associated with poor prognosis because of increased recurrence and reduced survival. Hence the carcinomas with higher expression of MF might be more aggressive and have poor prognosis hence are recommended for aggressive treatment and close follow up. Our results showed that in comparison to non-metastatic lesions, metastatic lesions show an increase in the number of α-SMA positive myofibroblasts. A study by Kellerman, et al was in agreement to our findings wherein abundance of myofibroblasts in the overall stroma or at the invasive tumor front was significantly correlated to pathologically confirmed lymph node metastasis. Statistically significant difference was present in our study among the metastatic and non-metastatic group in both the sites i.e buccal mucosa and tongue.(Table 4 and Graph 3). Tumour needs may be fulfilled by MFs as they play a role in angiogenesis, collagen breakdown and further invasion by the production of metalloproteinases and suppression of the host immune response thereby they form a part of tumor milieu. Using a double immunostaining technique with epithelial membrane antigen(EMA) and α-SMA, Vered et al demonstrated the presence of tumor cells which underwent EMT in cases of human tongue carcinoma. Using the triple immunostaining procedure showing loss of expression of E-cadherin (found only in epithelial cells and is responsible for cell to cell junction) was thought as a frequent event among the carcinoma cells and provided support to it. Furthermore, an inverse process of mesenchymal to epithelial transition (MET) can be acheived by the highly active neoplastic phenotype whereby the carcinoma derived stromal cells attain epithelial characteristics and establish new. According to the tissue organization field theory, cells do not tend to be quiescent but are normally in a proliferative state. For the induction of carcinogenesis mutation in the epithelial and stromal cells as well as disturbed stromal-epithelial interactions may be equally responsible, and thus emphasizes the importance of the neoplastic microenvironment in oncogenesis. Stroma produces myofibroblasts as a result of inductive effect from the adjacent genetically altered epithelium (carcinomatous epithelium). The exact mechanism by which these cellular elements have their effects on stromal and epithelial tissue compartments needs to be further clarified however, more sophisticated techniques are suggested for it. Therapeutic

targeting of myofibroblasts, their by-products or factors responsible for their trans- differentiation of fibroblasts to them may be beneficial to OSCC patients supported by the event that our findings are confirmed by future investigations. Myofibroblasts are a relevant component in the stroma of oral cancer cells, although they are not present in all OSCC cases. Although few in number, in vitro studies indicate that myofibroblasts are involved in the contractile function during the invasion process of OSCC, probably as a result of the modulation of the expression of growth factors, cytokines, extracellular matrix components, and proteolytic enzymes. Additionally, clinical, pathology, and immunohistochemistry tests have correlated the presence of high myofibroblast counts in oral cancer cell stroma with local disease recurrence and reduced patient survival. Despite the importance of these findings, the limited number of studies on the topic calls for additional research so that the molecular mechanisms by which myofibroblasts impact the biological behaviour of oral SCC are further clarified. SCC tongue is associated with the highest rate of metastasis as compared with other tumor sites in the oral cavity. However, less work has been done in the evaluation of SMF in buccal mucosa (most common site for oral cavity SCC in India) and tongue in relation with the lymph node metastasis. Hence this study "Role of myofibroblast and its evaluation by alpha smooth muscle actin in squamous cell carcinoma of tongue and buccal mucosa in relation to lymph node metastasis**"** was planned to know the role of α-SMA expression in correlation with the lymph node metastasis. The results of the study demonstrated presence of α-SMA positive myofibroblasts in the stroma of OSCC and showed increased expression and distribution in metastatic cases than the non-metastatic cases irrespective of the anatomical location i.e buccal mucosa and tongue.

Major contributing factor in cancer progression as suggested by the recent trends in cancer research is the inclusion of the microenvironment. During the conversion of stimulated normal epithelial cells to cancer cells, the tumor inducing action of MF starts and continues through progression to metastasis. It could be explained by realizing the fact that the SMF are part of the tumor that contribute to its progression and that the malignant cells are in a dynamic state-of changing phenotypes toward a mesenchymal differentiation that the partial response to routine anticancer treatment approaches which is often seen in oral squamous cell carcinoma. Thus, future cancer therapies should not only on "conventional" cancer cells and they would have to target stromal constituents. Anti-MF drugs can be used as therapeutic targeting of MFs preventing their transdifferentiation from fibroblasts and may be beneficial in oral squamous cell carcinoma patients

Supplementary Files (Figures)

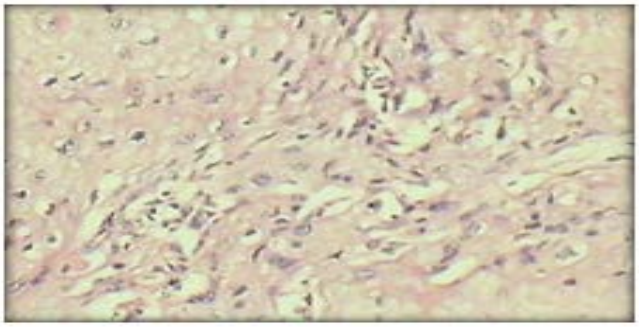


Figure 1. Allred Immunohistochemistry score

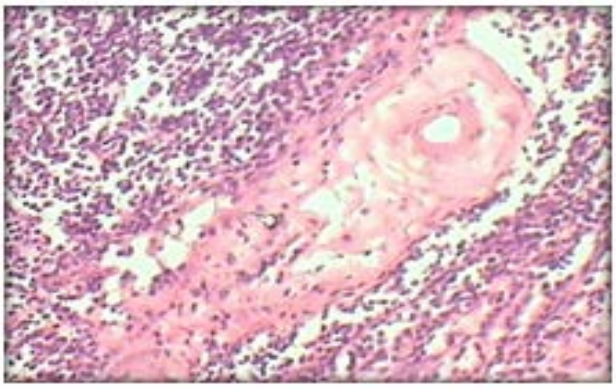
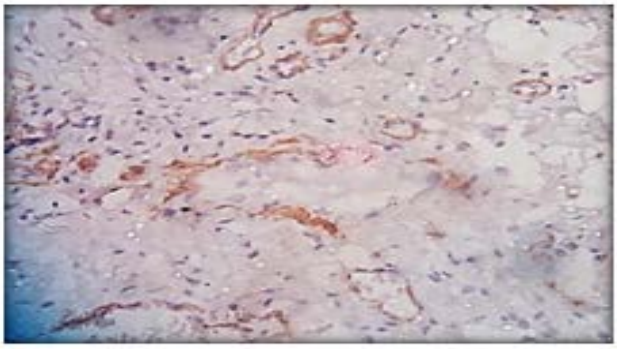
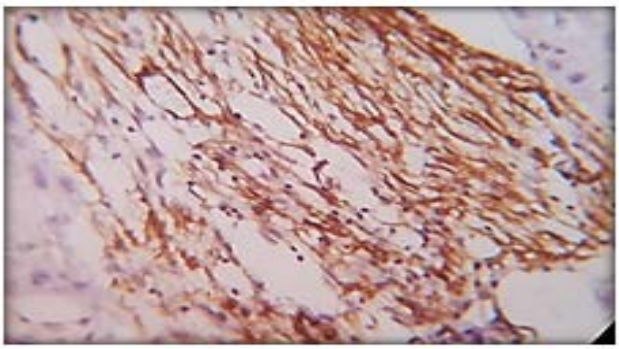


Figure 2: Photomicrograph showing squamous cell carcinoma with tumour cells showing dysplastic features (H&E, X400 magnification).



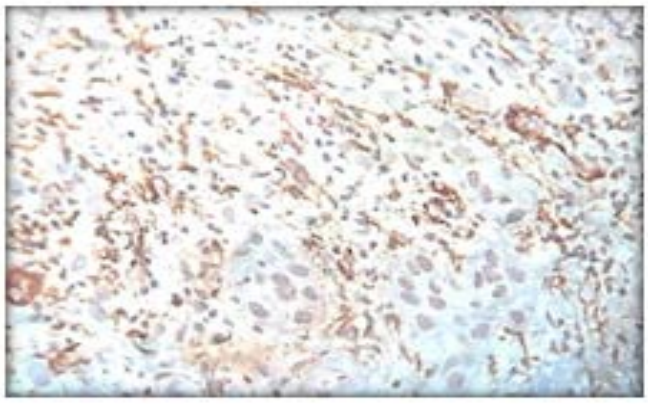
Metastatic lymph nodes

Figure 3: Photomicrograph showing squamous cell carcinoma with tumour islands metastasised into the lymph node (H&E, X400 magnification)



Internal control-blood vessels

Figure 4: Photomicrograph showing positive internal control- blood vessels in a squamous cell carcinoma case (DAB, X400 magnification)



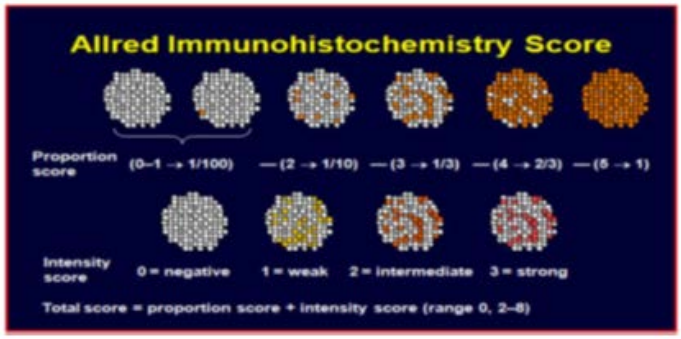


Figure 6: Photomicrograph showing α-SMA positive myofibroblast in a squamous cell carcinoma case without metastasis. Proportion score 3, Intensity score 1(DAB, X400 magnification).

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