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Original Article

Immunohistochemical Study of the Expression of Myofibroblasts in Metastatic Lymph Nodes of Oral Squamous Cell Carcinoma

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Abstract

Aim: Role of myofibroblasts in the stroma of oral squamous cell carcinoma (OSCC) is well studied, but activation of this entity in the metastatic lymph nodes is faintly known. The present study aims to evaluate the expression of myofibroblasts and to characterize the distribution pattern of these cells in metastatic lymph nodes of OSCC.

Materials and Methods: Sixty formalin-fixed paraffin-embedded tissue blocks of cervical lymph nodes (levels I, IIA, IIB, and III) of resected OSCC samples were examined immunohistochemically by anti-rabbit monoclonal α-smooth muscle actin. The expression of myofibroblasts was compared with clinicopathological parameters of OSCC.

Results: All metastatic lymph nodes of OSCC showed presence of activated myofibroblasts and expression of these cells was significantly ($p < 0.5$) increased in relation to pattern of invasion (POI) and grade of invasion in lymph nodes.

Conclusion: Myofibroblasts in lymph nodes may play a major role in establishing and supporting the growth of metastases in lymph nodes. The prognostic significance of myofibroblast expression in different POI in lymph nodes should be further investigated.

Key words: Alpha-smooth muscle actin, Lymph node, Metastasis, Myofibroblast, Oral squamous cell carcinoma

INTRODUCTION

Regional lymph node metastasis plays a pivotal role in initial diagnosis, staging, and management of oral squamous cell carcinoma (OSCC) and is the single most important prognosticator for afflicted patients.[1,2] Approximately 30% of patients with intraoral SCC present with positive regional lymph nodes.[3] Metastasis is driven by intrinsic factors such as the genetic and epigenetic characteristics of the cancer cells and critically affected by extrinsic factors mediated by the tumor microenvironment.[4] Tumor microenvironment (TME) consists of the extracellular matrix, carcinoma-associated fibroblasts, immune-inflammatory cells, and endothelial cells.[5,6] Cancer-associated fibroblasts (CAFs) are tumor-associated fibroblasts with myofibroblast-like phenotype which is the main component of the tumor stroma, and these have recently been paid a large amount of attention due to the prominent roles that they play in cancer development, progression, and metastasis.[7-10] A variety of growth factors and inflammatory chemokines secreted by stromal myofibroblasts are involved in the remodeling of the tumor stroma, the regulation of the motility of cancer cells, and the induction of tumor cells toward phenotypes that are more resistant to chemotherapy.[11,12] Some studies have reported that stromal myofibroblasts are associated with a poor prognosis in oral cancers.[13] Although the role of myofibroblasts in the stroma of primary tumor is well studied, only indirect observations currently suggest that modification of the stromal architecture such as activation of myofibroblasts takes place during metastatic

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conditioning of the lymph node microenvironment.[14] A conditioning of the microenvironment in lymph nodes is required making them receptive and supportive metastatic niches for disseminating tumor cells.[15,16]

Modified immunological responses and remodeling of the vasculature are the most studied tumor-induced pre-metastatic changes in the lymph node microenvironment that promotes metastasis, whereas modification of the stromal architecture such as activation of myofibroblasts in the cervical lymph node metastases of OSCC is not studied widely. In normal lymph nodes, myofibroblasts are not found within the body of the lymph node, whereas studies indicate that myofibroblastic reactions are evident in metastatic disease of breast cancer and metastatic lymph nodes of colorectal carcinoma.[17,18]

Although the knowledge of myofibroblasts participation in lymph node metastasis is evolving, there is little work investigating the expression of myofibroblasts in metastatic lymph nodes of OSCC. Hence, the present study aims to investigate the expression of myofibroblasts in metastatic lymph nodes of OSCC.

MATERIAL AND METHODS

Study Sample

The retrospective observational study comprised 50 metastatic and ten non-metastatic formalin-fixed paraffin-embedded tissue blocks of cervical lymph nodes dissected from radical neck dissection of histopathologically confirmed OSCC were obtained from the tissue archives of Department of Oral Pathology and Microbiology, SCB Dental College and Hospital, Cuttack. All the patients were informed about the study and written consent was obtained. The study was approved by the Institutional Research Ethics Committee vide no-IEC/SCBDC/012/2018.

Hematoxylin and Eosin Staining

All the samples were stained with hematoxylin and eosin with standard protocol and viewed under a light microscope to confirm and validate the prior diagnosis and grading. Lymph node parameters such as pattern of invasion (POI) and lymph node invasion grades were also evaluated as follows.

POI in Metastatic Lymph Nodes (as per Chandavarkar *et al.*[19])

Cords – Metastatic cells invade lymph nodes in the form of thin cords (Figure 1a and b), Islands – Metastatic cells invade lymph nodes in the form of small and large islands (Figure 1c and d),

Total replacement – Total lymph node effacement (Figure 1e and f).

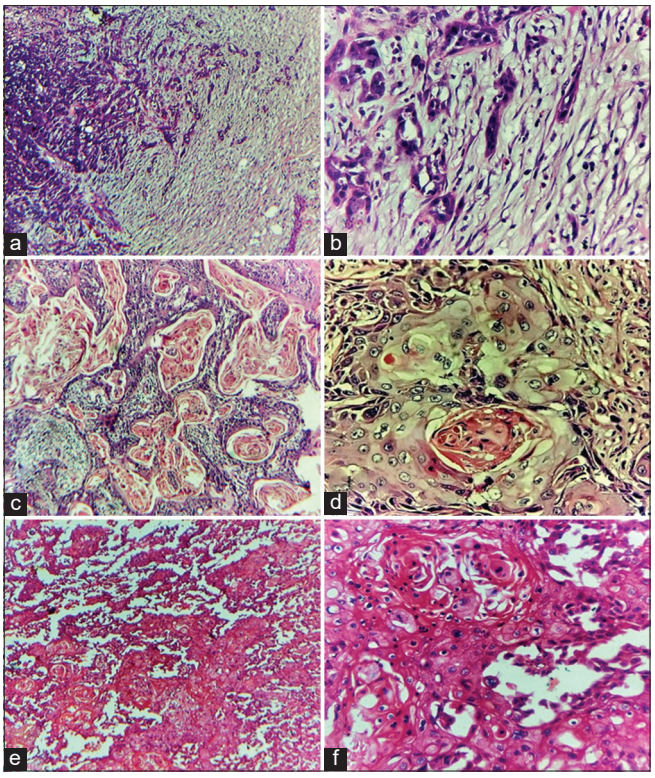

Figure 1: Pattern of invasion of cancer cells in cervical lymph nodes of oral squamous cell carcinoma. (a and b) The form of cords (H and E, ×100 and ×400); (c and d) in the form of island (H and E, ×100 and ×400); (e and f) in the form of total replacement (H and E, ×100 and ×400)

Grading of lymph node invasion (as per Don *et al.*[20]).

- Grade 1 – Micro-metastasis,
- Grade 2 – <50% of lymph node involved with metastatic OSCC cells,
- Grade 3 – >50% of lymph node involved with metastatic OSCC cells, and
- Grade 4 – Extracapsular extension.

Immunohistochemistry (IHC)

All lymph nodes were immunohistochemically stained by monoclonal rabbit α-SMA antibody (PR003, PathnSitu, India). IHC was performed according to standard protocols. Paraffin-embedded sections were deparaffinized in xylene twice for 5 min and hydrated in ethanol with a gradual concentration of 100%, 95%, and 70% for 3 min each. Slides were rinsed in distilled water. Antigen retrieval was performed using retrieval solution (PathnSitu pH 9 at 100 1C for 20 min) followed by cooling to room temperature for 1 h and washed 2 times with 0.1% phosphate buffered saline tween (PBST) for 3 min each. Slides were blocked at room temperature for 10 min using 0.3% H2O2 prepared with 1 × PBS. Slides were next incubated at room temperature for 30–40 min with α-SMA antibody (PathnSitu, India, Product code- PR003, 1:100). The secondary antibody used was HRP-conjugated

and was revealed by DAB (PathnSitu, India, Product code- OSH001). Serial sections obtained from non-metastatic lymph nodes were stained by CK 5/6 cocktail antibody (PathnSitu, India, Product code- PR106) following standard method. Images were obtained using a trinocular microscope (Lawrence and Mayo, equipped with a camera of 5 MP). Images were captured at ×400 magnification for evaluation.

Quantification of α-SMA IHC

As per the standard protocol of scoring, 10 random high-power fields were selected each for all samples (×400; Lawrence and Mayo, India) and analyzed independently by two observers. The following intensity scores (IS) were attributed according to degree of staining: Score 0, absence of staining; score 1, weak staining; score 2, moderate staining; and score 3, strong staining. The proportion score (PS) was attributed as per the percentage of stained cells (0, 0–5%; 1, 5–25%; 2, 25–50%; and 3, >50%). Multiplication of the intensity score (0–3) and proportion score (0–3) gives the overall staining intensity in a range 0–9. Average of scores of 10 fields was considered as the final staining score of the sample. The immunoreactivity was divided into three groups on the basis of final score.

Total score:

- <3 = Low positive
- 3–6 = Positive
- 6 ≥ = High positive

Statistical Analysis

The data were compiled and statistically analyzed using SPPS software version 21.0 (SPSS, Inc. IBM, USA). A comparison of α-SMA expression with various clinicopathological parameters such as age, gender, stage of primary tumors, POI in lymph nodes, and invasion grades of metastatic lymph nodes was done by the Chi-square test. ANOVA was employed to compare α-SMA expression with tumor differentiation and sites of primary tumor. Kappa statistics was performed to detect interobserver variability. *P* value of 0.05 or less was considered to be statistically significant.

RESULTS

Immunohistochemical Expression of α-SMA Positive Myofibroblasts in Metastatic Lymph Nodes

All metastatic lymph nodes (N = 50) showed positive expression of myofibroblast identified by α-SMA antibody in contrast to lack of expression in the body of non-metastatic lymph nodes (N = 10). The capsule of all lymph nodes showed a positive expression.

Expression of α-SMA Positive Myofibroblasts in the POI in Lymph Nodes

Higher expression of myofibroblast was detected in total replacement followed by cords and islands. Chi-square test revealed a highly significant difference in α-SMA expression in different lymph node invasion patterns ($p < 0.001$) [Table 1 and Figure 2a].

Expression of the Myofibroblasts in Different Grades of Lymph Nodes Invasion

A significant difference ($p < 0.001$) in the expression of the myofibroblasts in the different grades of lymph nodes invasion was noted. Grade 4 invasion cases showed more high positive expression than in Grade 2, where the high positive scores were the lowest [Table 1 and Figure 2b].

Distribution Pattern of the Myofibroblast in Metastatic Lymph Nodes

Three different patterns of myofibroblast distribution in metastatic lymph nodes were observed- focal, spindle, and network [Figure 3b-d]. Predominant network (36%) along with an equal percentage of focal and spindle (32%) patterns were observed. Statistically no significant

Table 1: Comparison of myofibroblasts expression with various pathological parameters of OSCC (χ^2)

Variables	Myofibroblasts expression			χ^2	*P*
	Low positive, *n* (%)	Positive, *n* (%)	High positive, *n* (%)		
POI					
Island	11 (61.1)	10 (55.6)	1 (7.1)	28.666	0.001*
Cords	6 (33.3)	0	4 (22.2)		
Total replacement	1 (5.6)	4 (22.2)	13 (92.9)		
Lymph node invasion grade					
Grade II	15 (83.3)	5 (27.8)	1 (7.1)	34.659	0.001*
Grade III	3 (16.7)	12 (66.7)	5 (35.7)		
Grade IV	0	1 (5.6)	8 (57.1)		
Pattern of myofibroblast					
Focal	5 (27.8)	5 (27.8)	6 (42.9)	5.693	0.223
Spindle	8 (44.4)	7 (38.9)	1 (7.1)		
Network	5 (27.7)	6 (33.33)	7 (38.88)		

* p=.001 is significant

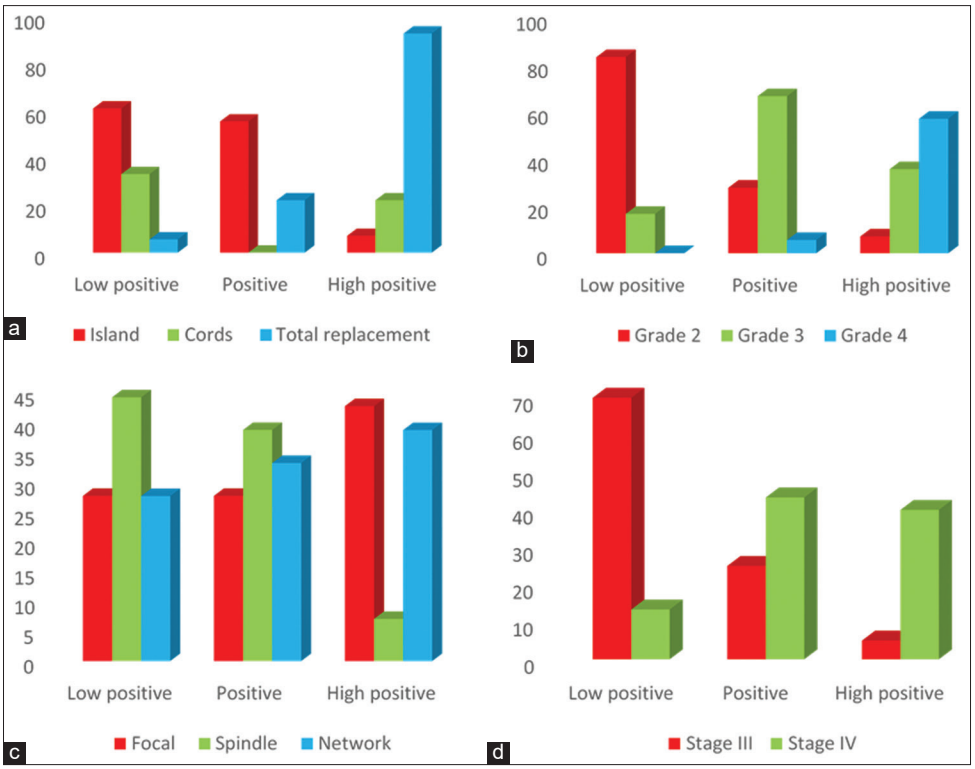

Figure 2: (a) Comparison of myofibroblasts expression in different pattern of invasion in lymph nodes revealing highest expression in total replacement; (b) comparison of myofibroblasts expression with grades of invasion in lymph nodes showing more high positive expression in grade IV cases; (c) comparison of pattern of myofibroblasts; and (d) comparison of myofibroblasts with tumor stage showing Stage IV tumor expressing more high positive and positive expression than Stage III tumor

difference of α-SMA expression in different distribution patterns ($P = 0.223$) was noted [Table 1 and Figure 2c].

The expression of myofibroblast was compared with different parameters like the age, gender, site, size and tumor differentiation which did not show significant differences [Tables 2 and 3]. A significant difference was noted in Stage IV cases than in Stage III cases ($p < 0.001$).

DISCUSSION

One of the key critical events in metastasis is pre-metastatic conditioning of lymph node micro-environment. However, there is a lack of extensive work investigating the participation of stromal cells in the induction of tumor microenvironment in cervical lymph nodes. To the best of our knowledge, the present study is only one of the few to report presence of myofibroblasts in cervical lymph nodes containing OSCC metastases and perhaps the first to compare its expression with the POI and grade of lymph node invasion.

In the present study, it is broadly observed that myofibroblasts, as identified by α-SMA positivity, occur abundantly within body of lymph nodes in agreement with Vered *et al.*[21] study containing OSCC metastases. This is in complete contrast to uninvolved lymph nodes of OSCC cases, where no internal myofibroblasts are seen (Figure 3a). Hence, it can be strongly assumed that the activation and recruitment of the myofibroblasts are brought about by the metastatic cancer cells into the lymph nodes. Cancer cells induce a favorable microenvironment that supports their existence and growth in lymph nodes similar to the primary tumor. The capsule in normal lymph node contains myofibroblasts, but the internal structure is devoid of such cells, the activation of which in metastatic lymph nodes is not a passive reaction.[18,21] A plausible explanation is that the capsular myofibroblasts provide specific attachment or anchorage point to the infiltrating cancer cells, thus helping them to establish themselves in the lymph nodes.[18] It is presumed that the capsule is a source of the activated myofibroblasts encountered in lymph node metastases of colorectal carcinoma.[18] Other sources of activated myofibroblasts like bone marrow is also hypothesized.[22]

Myofibroblast expression was, further, corelated with different patterns of lymph node invasion determined as island, cords, and total replacement types. The predominant pattern was found to be islands which is in agreement with previous study.[19] Highest expression of myofibroblast was observed (92% high positive) in total replacement

Table 2: Comparison of myofibroblasts expression with various clinical parameters of OSCC (χ^2)

Variables	Myofibroblasts expression			χ^2	*P*
	Low positive, *n* (%)	Positive, *n* (%)	High positive, *n* (%)		
Age					
<50 years	5 (33.33)	4 (26.66)	6 (40)	3.154	0.432
≥50 years	11 (31.42)	9 (25.71)	15 (42.85)		
Gender					
Male	13 (37.1)	11 (31.4)	11 (31.4)	4.637	0.327
Female	5 (35.7)	7 (50)	2 (14.3)		
Tumor size					
<5 cm	15 (38.46)	15 (38.46)	9 (23)	2.131	0.345
≥5 cm	3 (16.7)	3 (16.7)	5 (35.7)		
Tumor stage					
Stage III	14 (70)	5 (25)	1 (5)	18.122	0.001*
Stage IV	4 (13.33)	13 (43.33)	12 (40)		

OSCC: Oral squamous cell carcinoma; p=.001 is significant

Table 3: Comparison of tumor differentiation and intra oral sites of primary tumor with myofibroblasts expression in lymph nodes of OSCC (ANOVA)

Source of variation	Sum of squares	Df	Mean square	*F*	*P*
Tumor differentiation					
Between groups	1012.140	5	202.428	0.885	0.499
Within groups	10063.860	44	228.724		
Intraoral tumor site					
Between groups	0.118	3	0.070	0.125	0.779
Within groups	26.90	44	0.473		

OSCC: Oral squamous cell carcinoma

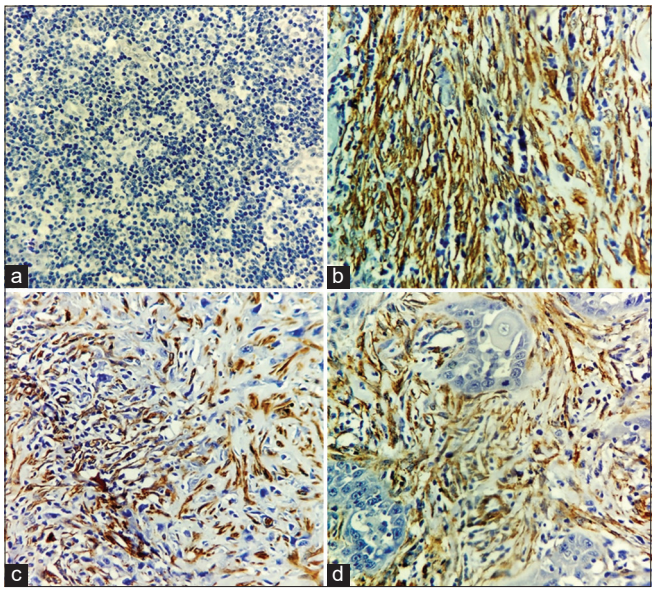

Figure 3: (a) Non-metastatic lymph node showing no expression of α-SMA positive myofibroblasts and (b-d) metastatic lymph nodes showing network, focal, and spindle arrangement of myofibroblasts (immunohistochemistry, ×400)

followed by cords (22.2% high positive) and islands (7.1% high positive) and these association was found to be highly significant ($P < 0.001$) [Table 1]. This study is the first to compare myofibroblast expression with POI in lymph nodes. The present study stated that an increased myofibroblasts might facilitate cancer cells in establishing and causing total effacement of lymph nodes. Further investigations are needed to confirm this fact.

An assessment of the distribution of grade of invasion in metastatic nodes was also made which revealed grade 2 invasion as predominant (42%), followed by grade 3 (40%) and grade 4 (18%). The expression of myofibroblast was highest in grade 4 (57.1% high positive), followed by grade 3 (35.7% high positive) and grade 2 (7.1% high positive) [Table 1]. A highly significant increase in myofibroblasts expression was noted with increasing lymph node grade ($p < 0.001$). That means myofibroblasts expression increases with the extent and volume of metastatic OSCC cells in lymph nodes. Yeung *et al.*[18] observed an increase in myofibroblasts expression in metastatic lymph nodes of colorectal carcinoma and it significantly correlated with the size of the metastasis. The increased expression of myofibroblasts with lymph node grading warrants that the metastatic OSCC cells are still dependent on their microenvironment. The association of myofibroblast expression in micrometastasis could not be investigated, because no micrometastasis was detected in lymph nodes under this study as ascertained by serial sectioning of negative lymph nodes and subsequent immunostaining with cytokeratin. It may be due to the inclusion of a small number of negative lymph nodes ($n = 10$).

On the evaluation of the distribution pattern of myofibroblasts, α-SMA expression was observed mostly between and around the neoplastic islands in lymph nodes. Myofibroblasts expression was also evident in the capsule of both metastatic and non-metastatic lymph nodes. A similar expression of myofibroblasts was found in the previous studies.[18,21] In this study, three different

patterns of myofibroblasts distribution in metastatic lymph nodes were observed – focal, spindle, and network (Figure 3b-d). Network pattern, predominated (36%) along with an equal percentage of focal, and spindle (32%) patterns were observed. A similar pattern of distribution of myofibroblasts was noted in the primary OSCC (Vered *et al.*[21], Alka *et al.*[23], and Smitha *et al.*[24]), but hitherto not explored in metastatic lymph nodes. A significant difference ($P = 0.223$) [Table 1 and Figure 2c] in myofibroblasts expression between these patterns has not been reached in the present study. Khalid *et al.*[25] showed that network pattern was more discerned often in poorly differentiated carcinomas. It is likely that neoplastic lesions show more invasive behavior and poorer prognosis due to the higher number of myofibroblasts arranged in network pattern.[26] The present study could not find such an observation and that might be due to a small number of PDSCC samples and a lack of follow-up data.

The expression of α-SMA positive myofibroblasts in various stages of the tumor was also compared. While all 50 metastatic lymph nodes were obtained from Stage III and Stage IV cases of OSCC, a significant difference in myofibroblast expression between Stage III and IV OSCC was observed ($P < 0.001$) [Table 2]. The above finding could be explained by the fact that Stage IV OSCC cases showed higher grade of lymph node invasion than Stage III cases [Figure 4] and myofibroblast expression increases with lymph node invasion grade. This could be one of the reasons for the higher expression of myofibroblasts in Stage IV cases.

However, the expression of myofibroblasts with other tumor related parameters such as the intraoral site, size, tumor differentiation, age, and gender of the patient was of no significance [Table 2].

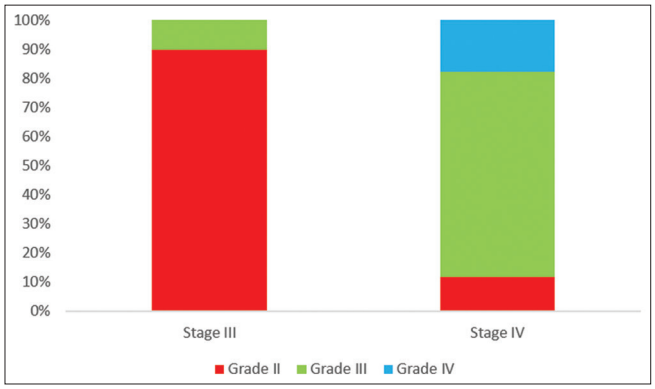

Figure 4: Percentage distribution of lymph node invasion grades in Stage III and Stage IV oral squamous cell carcinoma

CONCLUSION

The presence of activated myofibroblasts in lymph nodes and their increased expression with lymph node invasion grades and POI highlights the importance of the microenvironment in supporting cancers, even in the late metastatic stages. Further, understanding of the interaction between myofibroblasts and metastases may provide novel therapeutic targets for OSCC.

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