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EVALUATION AND CORRELATION OF DENSITY OF TUMOUR ASSOCIATED MACROPHAGES IN ORAL SQUAMOUS CELL CARCINOMA WITH OR WITHOUT LYMPH NODE INVOLVEMENT: A RETROSPECTIVE IMMUNOHISTOCHEMICAL ANALYSIS

Oncology

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ABSTRACT

Objective: To immunohistochemically evaluate and correlate the density of tumour associated macrophages (TAMs) in Oral Squamous Cell Carcinoma (OSCC) cases with or without lymph node involvement.

Material and Methods: This retrospective study was conducted on formalin fixed paraffin embedded tissue blocks of OSCC cases (n=30) treated with neck dissection, which were retrieved from the archives of the Department of Oral Pathology and Microbiology. OSCC cases (n=30) were classified based on pathological lymph node status as with lymph node metastasis (pN+) (n=15) and without lymph node metastasis (pN-) (n=15). Immunohistochemical analysis was carried out using immunohistochemical marker CD68 for TAM. The density of CD68+ TAMs in primary tumour (OSCC) was evaluated and correlated with pathological lymph node status.

Results: The mean density of TAMs (CD68+) was increased in pN+ OSCC cases when compared to pN- OSCC cases. Statistically this difference was significant.

Conclusion: The result obtained suggested that the mean density of TAMs (CD68+) may have a predictive value in determining the metastatic potential of OSCC.

KEYWORDS

CD68, Macrophages, Oral squamous cell carcinoma, Pathological lymph node status, Tumour Associated Macrophages, Tumor microenvironment.

Introduction:

Oral squamous cell carcinoma (OSCC) comprises more than 95% of all Oral cancers.[1] It is estimated to be the sixth most common cancer and in India the prevalence is around 45%.[2,3] Despite the significant advances in therapeutic strategies, the five year survival rate is only 53%.[4] In addition, a high percentage of patients have a poor response to therapy and high recurrence rates.[5] Hence, there is a need to identify novel biological markers that predict patients at high risk of disease.

Carcinogenesis or Cancer development is a multistep process which can be summed up into 3 stages - initiation, promotion and progression.[6] Progression includes metastasis of oral cancer, which is a complex process involving detachment of cells from tumour tissue, regulation of cell motility, invasion, proliferation and evasion through the blood vessels or lymphatic system.[7] Locoregional lymph node metastasis is considered as one of the significant independent prognostic factors in OSCC.[8] One of the key factors in lymph node metastasis is tumour microenvironment.[9] Tumor microenvironment contains diverse cells including tumor cells and various population of stromal cells (non- neoplastic cells) such as fibroblasts, epithelial cells, endothelial cells and infiltrating immune cells (innate and adaptive), as well as the products of these cells such as growth factors, extracellular matrix, chemokines, cytokines, enzyme and various metabolites.[10] In tumor microenvironment, inflammatory immune cells such as macrophages are referred to as Tumour Associated Macrophages (TAMs)/ Tumor Infiltrating Macrophages/ Panmacrophages.[11,12]

Many studies have shown an association between mean density of TAMs and prognosis in a variety of human cancers. In patients with breast, oral, thyroid, gastric, uterine and bladder cancer, a high density of infiltrating TAMs are associated with poor clinical outcomes while, in patients with prostate, colorectal and brain cancer, a high density of infiltrating TAMs have been associated with increased survival and improved prognosis.[12,13,14,15] In oral cancer, however, TAMs infiltration correlated with lymphangiogenesis, increased lymph node metastasis and advanced stages of tumour invasion and consequently enhanced tumour aggressiveness.[16,17]

Very few studies are done in OSCC to evaluate and correlate the mean density of TAMs with pathological lymph node status. Thus, the purpose of present retrospective study was to immunohistochemically evaluate and correlate the mean density of TAMs in tumor microenvironment of OSCC cases with pathological lymph node status using the immunohistochemical marker Cd68.

Material and Methods:

This retrospective study was conducted on formalin fixed paraffin embedded tissue blocks of OSCC cases (n=30) treated with neck dissection which were retrieved from the archives of the Department of Oral Pathology and Microbiology, MGM Dental College and Hospital, Navi Mumbai. The study was carried out from December 2014 to August 2016. Based on pathological lymph node status OSCC cases were categorized as those with lymph node metastasis (pN+) (n=15) and without lymph node metastasis (pN-) (n=15). (Figure 1 and 2) Recurrent cases of OSCC were excluded from the study.

Sections from tumor proper of each case were subjected to immunohistochemical staining technique to detect TAMs using prediluted primary antibody against CD68 (Dako, USA).[18] The presence, distribution and density of TAMs (CD68+) in OSCC microenvironment were evaluated by using conventional light microscopy. The mean density of TAMs (CD68+) for each case in tumor proper, was calculated as the mean density of infiltrating macrophages in five chosen HPFs.[19] (Figure 3) All collected data was entered into SPSS 16.0 (statistical package for social sciences version 16.0) worksheet. Further analysis was performed using statistical test such as Independent student's't' test. A significance level of 0.05 was applied to decide the statistical significance of the hypothesis being tested.

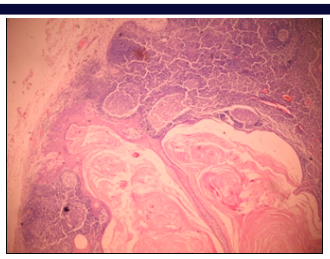


Figure 1: Photomicrograph of H & E stained soft tissue section showing pN(+) lymph node. [Haematoxylin and Eosin, 40x]

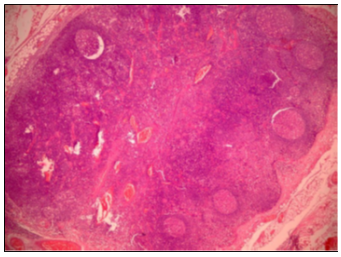


Figure 2: Photomicrograph of H & E stained soft tissue section showing pN(-) lymph node. [Haematoxylin and Eosin, 40x]

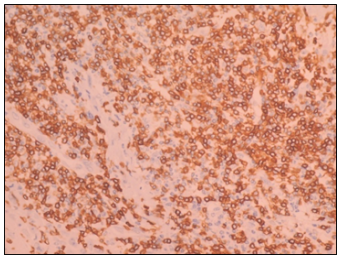


Figure 3: Photomicrograph of immunohistochemically stained soft tissue section of OSCC in primary tumor showing immunoexpression of CD68+ Tumour associated macrophages. [Immunohistochemistry,400x]

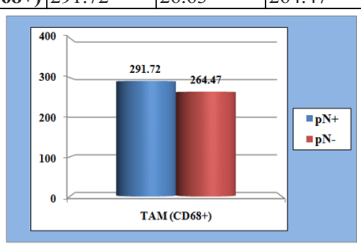
Results:

Immunohistochemical analysis was carried out on all histopath ologically diagnosed cases of OSCC (n=30) with lymph node metastasis pN+ (n=15) and without lymph node metastasis pN- (n=15). On immunohistochemical evaluation the mean density of TAMs (CD68+) in the tumor microenvironment ranged from 191.2 to 327.6. The descriptive analysis showed that the mean density of TAMs (CD68+) in pN+ cases was 291.72 and in pN- it was 264.47. (Table 1) This showed that there was an increase in the mean density of TAMs (CD68+) in pN+ OSCC cases when compared to pN- OSCC cases. (Graph 1)

Independent student's't' test showed that there was a statistically significant difference in the mean density of TAMs (p=0.016) in pN+ and pN- cases of OSCC. (Table 2)

Table 1: Comparative evaluation of the mean density of TAMs (CD68+) in pN+ and pN- cases of OSCC

	Mean density of TAMs and its phenotypes in pathological lymph node status			
	pN+		pN-	
	Mean	Standard Deviation	Mean	Standard Deviation
TAMs (CD68+)	291.72	26.63	264.47	31.27



Graph 1 compares the mean density of TAMs (CD68+) in pN+ and pN- cases of OSCC. An increase in the mean density of TAMs (CD68+) was seen in pN+ OSCC cases when compared to pN- OSCC cases.

Table 2: Statistical correlation of the mean density of TAMs (CD68+) in pN+ and pN- cases of OSCC
Independent t-test results:

	Pathological Lymph Node Status (pN)	n	t-stat	df	p-value	Mean difference
TAMs (CD68+)	pN (+)	15	2.570	28	0.016*	**27.253**
	pN (-)	15				

*. The p value is significant at the 0.05 level.

Discussion:

The present retrospective study was conducted on formalin fixed paraffin embedded tissue blocks of OSCC cases (n=30) treated with neck dissection, which were retrieved from the archives of the Department of Oral Pathology and Microbiology. Based on pathological lymph node status OSCC cases were categorized as with lymph node metastasis (pN+) (n=15) and without lymph node metastasis (pN-) (n=15). In our study, on statistically evaluating and correlating the mean density of TAMs (CD68+) in the sections of tumor proper with pathologic lymph node status, we found that the mean density of TAMs (CD68+) in cases with pN(+) (n=15) was 291.72 (SD= 26.63) and in cases with pN(-) (n=15) it was 264.47 (SD= 31.27). We found a higher mean density of TAMs (CD68+) in pN(+) than in pN(-) cases of OSCC and this difference was statistically significant. (p= 0.016). Our result was in accordance with Costa NL et al[15], Liu SY et al[17], Lu CF et al[19], He KF et al[20], Hu Y et al[21] and Li C et al[22] who found that density of TAMs (CD68+) was significantly higher in OSCC cases with lymph node metastasis than without lymph node metastasis. Marcus B et al[16] observed that high levels of TAMs infiltration significantly correlated with lymph node metastasis and extracapsular lymph node spreading in oral cancer. Fujii N et al[23] in their study on OSCC observed that there was no statistical significant association of high numbers of TAMs (CD68+) with lymph node metastasis. Lin JY et al[12] observed higher density of intratumoral TAMs in the lymph node metastasis group of supraglottic laryngeal squamous cell carcinoma. Sun S et al[24] observed that high expression of TAMs (CD68+) in laryngeal Squamous Cell Carcinoma was significantly correlated with pathological lymph node metastasis. Ding M et al[25] observed TAMs (CD68+) were higher in the lymph node metastasis positive than lymph node metastasis negative cases of breast cancer. Chen et al[26] found that high TAMs (CD68+) were significantly associated with lymph node metastasis in pancreatic ductal adenocarcinoma. Zhang B et al[27] in their study on lung adenocarcinoma and Qing W et al[28] in their study on papillary thyroid carcinoma found statistically significant correlation of TAMs (CD68+) with lymph node metastasis. Schoppmann SF et al[29] in their study observed significant association of VEGF-C and VEGF-D producing TAMs with lymph node metastasis and they suggested that in human cervical cancer, the VEGF-C released by TAMs plays a novel role in peritumoral lymphangiogenesis and subsequent lymphatic metastases.

TAMs promote tumor lymphangiogenesis and lymph node metastasis through paracrine and cell autonomous modes. The paracrine mode consists of the expression of variety of pro-lymphangiogenic factors (VEGF C and VEGF D) that activate the pre-existing lymphatic vessels.[8] In cell-autonomous mode tumor mobilization of macrophage-derived lymphatic endothelial cell progenitors (M-LECP) integrate into lymphatic vessels prior to sprouting.[8] According to Kerjaschki D et al[30] TAMs induce lymphangiogenesis which is an initial step for lymph node metastasis in two different ways, either by stimulating the division of preexisting local lymphatic endothelial cells or by transdifferentiating and directly incorporating into the endothelial layer. Guruvayoorappan C et al[31] suggested that MMP2 and MMP9 secreted by TAMs and tumor cells degrade the proteins in the extracellular matrix to promote metastasis. In addition, MMP7 secreted by TAMs and tumor cells also promote tumor metastasis through converting the receptor activator of nuclear factor kB ligand (RANKL).[32,33] TGF-b and IL-10 secreted by TAMs contributes to inhibition of Th1 immune cells and cytotoxic T lymphocytes (CTLs) (primary effector cells for an efficient anti tumor immunity), while promoting the generation of immunosuppressive Foxp3+ Tregs cells which promote the tumor growth, progression and metastasis.[34]

Very few studies were done in OSCC to evaluate and correlate, the

TAM (CD68+) with pathological lymph node status. In our study on OSCC, we found that the mean density of TAMs (CD68+) was higher in lymph node metastasis cases as compared to without lymph node metastasis indicating that increase in mean density of TAMs (CD68+) has a key role in tumor progression and lymph node metastasis. However, more research work on a much larger sample size would further authenticate our observation.

Conclusion:

It is apparent from the results of present study that TAMs (CD68+) could be considered pro-tumour as they have a key role in tumor growth, tumor progression and metastasis. Further studies with larger sample size are required to ascertain the interaction between TAMs and cancer cells. This will emphasize the role of TAMs as an effective biomarker in predicting lymph node metastasis which is an independent prognostic marker and certainly shed new light on the development of efficient targeted anticancer therapy.

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Conflicts of interest:

There are no conflicts of interest.

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