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# Quantification Of Tumor Associated Macrophages And Lymphatic Vessels With Their Association In Oral Squamous Cell Carcinoma.

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**ABSTRACT**

The survival of the patients with oral squamous cell carcinoma remains unaffected despite recent therapeutic advances. Macrophages proliferate, differentiate or become activated under the effect of interleukins or growth factors. These activated macrophage ceases to respond to proliferative stimuli. At this circumstance they have a harmful rather than repairing effect. Hence, it has been hypothesized that tumor-associated macrophages (TAMs) may play crucial role in tumor aggressiveness. This study aims to quantify the interrelationship of tumor associated macrophages and lymphatic vessels in oral squamous cell carcinoma using Immunohistochemical markers CD68 and Podoplanin respectively which may provide us with vital information regarding the role of TAMs in the biological behavior and lymphangiogenesis in OSCC. It was found that there was statistical significant association between tumor associated macrophage count and lymphatic vessels count (p value < 0.001). As tumor lymphangiogenesis is primary cause for lymph node metastasis that promote systemic spread and shorten patient survival. It can thus be of great importance as therapeutic depletion of macrophages can be used as an adjuvant treatment in cases of OSCC.

**Keywords:** Tumor-associated macrophages, CD68, Podoplanin, Oral squamous cell carcinoma

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## INTRODUCTION

The head and neck malignancy is the first leading cause of death in South-Asia.[1] There is high prevalence of oral and oro-pharyngeal carcinomas in Asian countries like India with up to half of all malignancies mainly due to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and betel quid chewing .[2]

The existence of an inflammatory microenvironment in all tumors and the critical role played by inflammation in tumorogenesis became clearly evident and got acceptance only in the last decade. Neutrophils in the initial stages of inflammation and later on macrophages and lymphocytes initiates the mechanism of acquired immunity. Macrophage activity then switches from pro-inflammatory to anti-inflammatory which play a key role in innate immune response and form bridge between innate and acquired immune response. Macrophages proliferate, differentiate or become activated under the effect of interleukins or growth factors. This activated macrophage ceases to respond to proliferative stimuli. At this circumstance they have a harmful rather than repairing effect.[3]

CD68 (Cluster of Differentiation 68) is a glycoprotein which binds to low density lipoprotein. It is expressed on macrophages and cells of their lineage such as monocytes, histocytes etc. CD68 has been widely used as a pan-macrophage marker for tumor-associated macrophages (TAM) which always involve in carcinogenesis.[4]This bio-marker can be used to identify TAMs in oral cancers such as oral squamous cell carcinomas (OSCC). Podoplanin is a mucin-like glycoprotein that is important in lymphangiogenesis detected on the surface of podocytes belonging to the family of type-1 sialomucin transmembrane glycoproteins.[5] Podoplanin expression was found in tumor cells of various types of cancer, such as vascular tumors, malignant mesothelioma, OSCC etc. [6] Literature has confirmed that Podoplanin expression is rather higher in early cancer stages than in more advanced ones.[7] The presence of this protein in tumor cells is useful for pathological diagnosis and seems to be expressed by aggressive tumors, with higher invasive and metastatic potential.

The angiogenic phenotype of macrophages is in part defined by their ability to secrete molecules that promote or inhibit angiogenesis. Depending on their activation state, M2 TAMs produce a variety of proangiogenic and lymphangiogenic growth factors, cytokines and proteases.[8,9,10] Even the stimulation of monocytes by tumor products such as interleukin 10 (IL-10) clearly drives monocytes into M2 angiogenic macrophages, secreting the highly angiogenic growth factor ( Vascular endothelial growth factors) VEGF. [11-15] Most TAMs found during human cervical carcinogenesis are of M2 type and express VEGF-C, VEGF-D, as well as the VEGFR-3, all of which are implicated in the formation of lymphatic vessels and finally, lymphatic metastasis.[9] TAMs preferentially accumulate in the hypoxic and necrotic regions within the tumor and become M2 angiogenic [16-18] However, how M2 TAMs respond to the oxygen level within the tumor mass is still unclear. It is admitted that the response of cells to hypoxia is mediated by the hypoxia inducible factor (HIF) system. [19]

The survival of the patients with OSCC remains unaffected despite recent therapeutic advances. Hence, it has been hypothesized that TAMs play crucial role in tumor aggressiveness so this study aims to quantify the interrelationship of tumor associated macrophages and lymphatic vessels in oral squamous cell carcinoma using Immunohistochemical markers CD68 and Podoplanin respectively which may provide us with vital information regarding the role of TAMs in the biological behavior and lymphangiogenesis in OSCC.

## MATERIALS AND METHODS

### Study Design

Archival specimens of formalin-fixed paraffin-embedded tissue blocks of OSCC patients were retrieved from the Department of Oral and Maxillofacial Pathology, Dr. D.Y.Patil Dental College, Pimpri, Pune. The study group comprised 30 samples which were divided into 2 groups. Group 1- OSCC with lymph node positive tissue (n=15) and Group 2- OSCC without lymph node positive tissue (n=15).

**Inclusion criteria**

Archival specimens of formalin-fixed paraffin-embedded tissue blocks of OSCC with lymph node positive tissue and without lymph node positive tissue.

**Exclusion criteria**

OSCC cases with history of any major systemic disease such as chronic renal failure, liver cirrhosis etc.

**Tissue processing and TMA preparation**

Histological slides of each specimen were prepared for IHC staining by cutting 3 µm tissue sections on a standard microtome. Immunohistochemical technique was performed using the avidin-biotin-peroxidase protocol. Antigen retrieval was performed with Target antigen retrieval solution pH 9 (Dako A/S, CA, USA) in a water bath, followed by incubation with 6% hydrogen peroxide to quench endogenous peroxidase. The sections were then incubated in blocking solution (3% bovine serumalbumin) for 1 hour at room temperature, followed by primary antibody incubation, previously diluted in blocking solution. Mouse Monoclonal CD68/ Macrophage marker Ab-3 with dilution of 1:40 and Podoplanin (D2-40) with dilution of 1:60 antibody was incubated for 30minutes at room temperature. Sections were exposed to the LSABTM system (DAKO A/S, CA, USA), developed in diaminobenzidine (Dako A/S, CA, USA) and counterstained in Mayer's hematoxylin. For the antibody, positive and negative controls were used. The slide were then observed under light microscope at 10X magnification for 5 'Hot spots' i.e. areas where density of CD68 positive cells (TAM) and Podoplanin positive cells (lymph vessels) were they wer maximum in number. Then these 'Hot spots' were observed in high magnification (40X) for counting.

**Method of data analysis**

Values were obtained after counting the number of macrophages in the three 'hot spots' regions and its average were taken. The average was taken and graded as GRADE +1: Low expression, GRADE +2: Moderate expression, GRADE +3: High expression. Similarly values were obtained after counting the number of lymph vessels in the five 'hot spots' regions and its average were taken. Values were subjected to statistical analysis using chi square test, ANOVA and post hoc Bonferoni test.

## RESULTS

After compiling the data according to inclusion and exclusion criteria samples were also graded as well differentiated and moderately differentiated within the samples of OSCC in the study. Statistical significant relation was found between different grades of OSCC and tumor associated macrophage count. In well differentiated OSCC, 10 (76.92%) tissue samples showed mild TAM count and 6 (60%) tissue samples showed moderate TMA count which was more in comparison to moderately differentiated OSCC with 3 (23.7%) tissue samples showed mild TMA count and 4 (40%) tissue samples showed moderate TMA count. Where as in well differentiated OSCC 1 (14.28%) tissue samples showed severe TMA count which was less when compared to 6 (85.71%) tissue samples showed severe TMA count in moderately differentiated OSCC.

Statistical significant relation was found between lymph nodes and tumor associated macrophage count. In positive lymph node samples, 3 (23.07%) tissue samples showed mild TAM count which was less when comparison to 10 (76.92%) tissue samples showed mild TAM count in negative lymph node tissue samples. 6 (60%) tissue samples showed moderate TMA count and 6 (85.71%) tissue samples showed severe TMA count in positive lymph node samples which was more when compared to 4 (40%) tissue samples showed moderate TMA count and 1 (14.28%) tissue samples showed severe TMA count in negative lymph node tissue samples ( Table 1).

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**Table 1: Association of lymph node with CD68 count**

| Lymph node | CD68 count | | |
|---|---|---|---|
| | Mild | Moderate | Severe |
| Positive | 23.07% | 60% | 85.71% |
| Negative | 76.92% | 40% | 14.28% |
| Total | 100% | 100% | 100% |
| Chi square value= 7.741; p value= 0.019 | | | |

It was observed that patients of OSCC with positive lymph node showed increased density of lymphatic vessels as compared to patients with negative lymph node. Results were statistical significant different grades of OSCC and lymphatic vessels count (p value 0.002) with mean value of lymphatic vessels count being higher in moderately differentiated OSCC (8.46±2.9) compare to mean value of well differentiated OSCC (5.24±3.3). Statistical significant association was found between lymph nodes and lymphatic vessels count (p value < 0.001) with mean value of lymphatic vessels count being higher in positive lymph node tissue samples (8.47±2.9) compare to mean value of negative lymph node tissue samples ( 4.80 ± 1.6) ( Table 2).

**Table 2: Association of lymph node with Podoplanin count**

| Podoplanin count | Lymph node | N | Mean | SD | t test | p value |
|---|---|---|---|---|---|---|
| | Positive | 15 | 8.47 | 2.9 | 4.147 | <0.001 |
| | Negative | 15 | 4.80 | 1.6 | | |

After applying one way Anova it was found that there was statistical significant association between tumor associated macrophage count and lymphatic vessels count (p value < 0.001) ( Table 3). To assess the difference between the groups post hoc test (Bonferoni) was applied and it was found that there was statistically significant difference between the groups with severe grades of tumor associated macrophage having high number of lymphatic vessels count followed by moderate and mild grades of tumor associated macrophage ( Table 4).

**Table 3: ANOVA- Association between tumor associated macrophage count and lymphatic vessels count**

| | Sum of Squares | Mean Square | F | P value |
|---|---|---|---|---|
| Between groups | 184.945 | 92.472 | 31.201 | <0.001 |
| Within groups | 80.022 | 2.964 | | |

**Table 4: Bonferoni- Associations of CD68 grades with Podoplanin count**

| CD68 | | Mean Difference | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound |
| Mild | Moderate | -2.769 | .002 | -4.62 | -.92 |
| | Severe | -6.341 | .000 | -8.40 | -4.28 |
| Moderate | Mild | 2.769 | .002 | .92 | 4.62 |
| | Severe | -3.571 | .001 | -5.74 | -1.41 |
| Severe | Mild | 6.341 | .000 | 4.28 | 8.40 |
| | Moderate | 3.571 | .001 | 1.41 | 5.74 |

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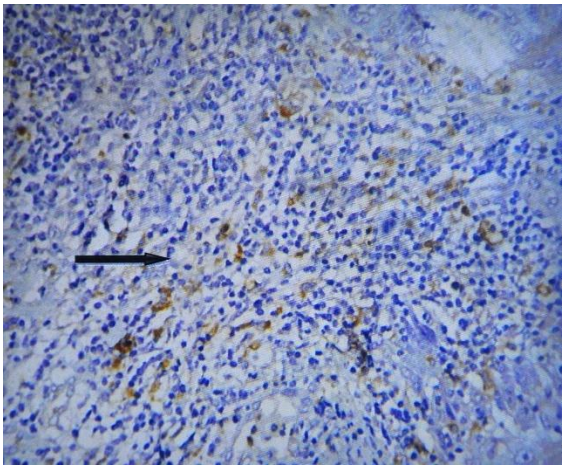

**Figure 1: High CD68 Count**

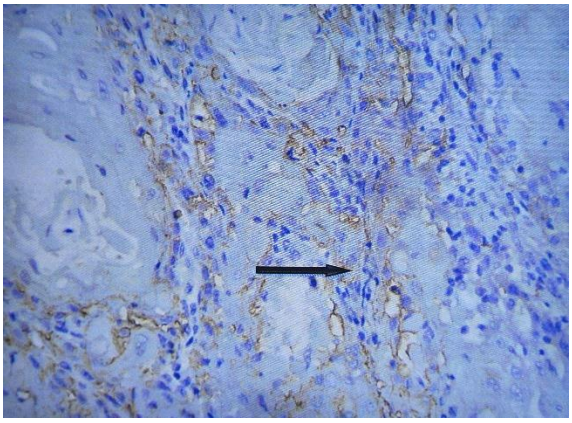

**Figure 2: High Phodoplanin Count**

## DISCUSSION

Cancer progression is a multistep process that consists of tumor growth invasion and metastasis. A recent concept is that cancer and inflammation are inherently linked.[20] Transition of TAM from an anti-tumor to a pro-tumorigenic type is characterized by high expression of factors that activate endothelial cells, suppress immune response, degrade extracellular matrix, and promote tumor growth. Cumulatively, these products of TAMs promote tumor expansion and growth of both blood and lymphatic vessels that facilitate metastatic growth.[3]

Both experimental and clinical studies have shown that TAMs significantly promote tumor lymphamgiogenesis through paracrine and cell autonomous modes.[21] A plethora of prior observations clearly show in addition to chronic inflammatory disease, macrophages also play a prominent role in tumor progression and metastasis. Tumor metastasis was previously attributed to the ability of tumor-associated macrophages (TAMs) to promote angiogenesis (i.e., the formation of blood vessels) that facilitate hematogenous spread. However, most epithelial malignancies metastasize first to the regional lymph nodes (LNs) through lymphatic vessels prior to dissemination through blood vasculature. These observation suggest that tumor macrophages play a critical role in promoting not only angiogenesis but also lymphangiogenesis that lead to lymphatic metastasis.[21]

Many inflammatory mediators have potent pro-lymphangiogenic properties. It is, therefore, not surprising that virtually all epithelial tumors include either intratumoral or peritumoral lymphangiogenesis and utilize newly-created lymphatic channels for metastatic spread.[21]

Our results showed that regions of maximum TAM positivity (hot spots) were found to be adjacent to the proliferating malignant epithelial islands. TAM count was significantly increased in OSCC with positive lymph node metastasis, more advanced clinical stages of recurrence. Our findings are in accordance with them as we also found significant macrophage count in moderately differentiated OSCC specimens and samples with lymph node metastasis.

Taiming Dai et al. investigated the significance and relationship between matrix metalloproteinase 9 (MMP-9) and infiltration of macrophages in the process of invasion and metastasis in OSCC. They found that there were masses of macrophage infiltration in OSCC, which is in agreement with their previous findings as well as the findings in our study. In their study a large quantity of macrophage infiltration was found in the matrix of OSCC and the zones of necrosis, but the MMP-9 expression mainly focused in the tumor cells, indicating that macrophages are not the main source of MMP-9 over-expression in the OSCC tissue. They also found that both MMP-9 and macrophages have a relationship with the TNM tumor staging and condition of the lymphatic metastasis. Furthermore, there was a positive correlation between the macrophage count and MMP-9 expression in the tumor. These findings suggest that there may be a cooperation between macrophages and MMP-9 in OSCC, which jointly promote the invasion and metastasis of the tumor.[22]

Lo Muzio L et al. studied the immunohistochemical expression of bcl-2, CD2, CD20, CD45 and CD68in paraffin-embedded OSCC specimens in order to establish their possible correlation with the degree of tumor differentiation. The most expressed molecule was CD68 and their results show a trend for the association of inflammatory infiltrate with the degree of tumor differentiation: well and moderately differentiated tumors tend to be associated with a dense inflammatory infiltrate while poorly differentiated cancers seem to be associated to a low inflammatory infiltrate. Similarly to their study we found significant difference in the expression of CD68 biomarker marker the various histopathological grades of OSCC.[23]

The presence of TAMs was found in all the OSCC specimens in our study and an increased TAM infiltration around the neoplastic and malignant epithelial islands and cells was seen. This may also suggest that the macrophages are involved in tumor cytotoxicity as well as scavenging of tumor cell debris.

It is becoming increasingly clear now from recent research that TAMs are composed of multiple distinct populations with overlapping features that depend on a variety of factors including location in the microenvironment, stage of the tumor, and type of cancer.[24] The macrophage balance hypothesis postulates that there are different net effects at different stages of tumor progression: in early stages of carcinogenesis, innate responses are beneficial to the host and involve the activation of effective surveillance by adaptive immunity to eliminate tumor cells, while in established malignancy TAMs orchestrate “smoldering inflammation” that promotes tumor progression.[25]

Podoplanin antibody Mouse monoclonal [D2-40] reacts with an O-linked sialoglycoprotein (MW 40kDa) found on lymphatic endothelium, and marks neolymphatic vessels in tissue samples.[26]

Podoplanin expression was evacuated in our study and correlated with different grades of carcinoma and lymph node status. We have found that podoplanin was overexpressed in moderately differentiated cases as compared to well differentiated OSCC and also showed significantly higher expression in cases of positive lymph node.

Gerhard Frank Huber et al studied 120 patients with HNSCC of oral cavity and oropharynx undergoing sentinel lymph node biopsy. They found that Podoplanin expression correlated significantly with SLN metastasis. Their findings support a role of podoplanin in tumor progression and lymphangeneous metastasis in vivo.[27] Our study also showed significantly higher expression of podoplanin in cases of positive lymph node. In contrast Sang Woon Lee at al demonstrated that there was no statistical difference in the expression of endoglin and podoplanin regardless of whether or not the lymph node was positive.[28]

Sachiko Saki et al analyzed 82 specimens of OSCC immunohistochemically using podoplanin. Multiple regression analysis revealed a significant relationship of strongly positive (>50%) expression of podoplanin to advanced clinical stage especially grade III. they found that the intensity of podoplanin expression in OSCC cells predicted the prognosis of patients. In our study we have also found that tumor indued lymphangiogenesis co related with lymph node metastasis.[29]

A study conducted by Zang G et al showed that Podoplanin in epithelia was increased with the exacerbated epithelial proliferation. High expression of Podoplanin in lesional epithelia accounted for 45% in oral leukoplakia , 81% and 78% in early and infiltrated stage of of squamous cell carcinoma. Podoplanin expression in infiltrated stage of squamous cell carcinoma was positively correlated with the lymph vessels density. [30]

De Sousa et al concluded that, the majority of cases with nodal involment presented a high peritumoral lymphatic vessel density (LVD). Microvessel density (MVD) was statistically associated with metastasis, and a significant association between the lymphangiogenic factors and the density of vessels in the intratumoral region was also seen. In our study we also found that a significant association between metastasis and density of vessels in the intratumoral region.[31] The well-differentiated tumors expressed decreased podoplanin in their study. Similar observation was seen as neolymphangiogenesis was more prominent in cases with lymph node metastasis in our study.

In recent years, evidence has accumulated that macrophages are not only critical regulators of angiogenesis, but also crucial participants in lymphangiogenesis , both in inflammatory settings in tumors.[21] Importantly, macrophages may simultaneously include both angiogenesis and lymphangiogenesis by the production of VEGF-A and MMP9. These cytokines which are abundantly produced by subpopulations of TAM were shown to induce the development of both blood and lymphatic vessels. Thus, TAM- derived factors can link tumor angiogenesis and lymphangiogenesis. [32]

Macrophages can utilize two main pathways to stimulate lymphangiogenesis: either by the direct secretion of prolymphangiogenic factors or by transdifferentiation into lymphatic endothelial cell, actively taking part in the formation of lymphatic vessels. [33]

Masahiro ohta et al examined whether macrophage infiltration is associated with neovascularity in esophageal carcinoma and observed a close positive correlation between the number of macrophages infiltrating the tumors and tumor vascularity. They also found macrophage infiltration to be positively correlated with malignant features such as tumor depth, lymphatic invasion, venous invasion and lymph node metastasis. They concluded that, MCP-1 expression by esophageal carcinomas is associated with macrophage infiltration and tumor vascularity. Interactions between cancer cells and macrophages appear to be important for tumor angiogenesis and progression. We too observed a positive correlation between macrophage count and lymphangiogenesis and demonstrated that macrophages have high potential to promote generation of new lymphatic vessels.[34]

Sarah J Storr et al investigated the role of vascular invasion (blood and lymphatic), vessel density and the presence of tumor-associated macrophages as prognostic markers in 202 cutaneous melanoma patients. A high macrophage count was significantly associated with the presence of any lymphatic vessel invasion, blood microvessel density and immunohistochemically determined vascular invasion, indicating the likely importance of macrophage in the process of tumor vascularisation and lymphatic dissemination. Therefore high microphage count may be associated with neovascularisation and primary tumor growth, and may also promote invasion through lymphatic vessels.[35] In our study it was observed that positive lymph node cases showed more TAM count as compared to negative lymph node cases. Moreover the TAMs were mostly located beside the malignant epithelial islands. Also statistical relation was found between TAM count and different grades of OSSC suggesting TAM as a prognostic factor.

Petr Buzrla et al focused on the lymphangiogenesis and its distribution and density at cutaneous melanomas and further on its correlation with the status of the sentinel lymph node and the VEGF expression by melanoma cells and by stromal cells. They observed that density of intratumoral and peritumoral lymphatic vessels

could not help to predit the potential of metastasis in the sentinel lymph node. Contradictory to this we have found Statistical significant association between lymph node and lymphatic vessels count with mean value of lymphatic vessels count being higher in positive lymph node cases compare to mean value of negative lymph node cases.[36]

Bicheng Zhang examined the density and prognostic significance of M2- polarized tumor associated macrophages in lung adenocarcinoma. Their data demonstrate that a shift toward the production of Th2 cytokines, a factor that includes alternative macrophage activation, occurs within the lung adenomacarcinoma tumor microenvironment. The result showed peritumoral lymphatic microvessel density was significantly higher in the high M2- polarized tumor-associated macrophage group than in the low M2- polarized tumor-associated macrophage group. A significant difference in overall patient survival was detected not only between patients with tumors with high and low macrophage counts but also between patients with tumors with high and low counts of M2-polarized macrophages. Hence they have concluded TAM in lung adenocarcinoma have an M2-polarized subtype subtype and are associated with poor prognosis, perhaps resulting from accelerated lymphangiogenesis and lymph node metstasis.[37]

Bi Cheng Zhanget al in another study examined the relationship between TAMs and lymphnagiogensis in the prognosis of patients with lung adenocarcinoma. A positive correlation existed between TAMs count and D2-40 positive peritumoral lymphatic microvessel density (LMVD). Both TAMs count and peritumoral LMVD were independent prognostic factors for overall survival. Their results indicated that TAMs infiltration correlation with tumor lymphangiogenosis and poor survival in lung adenocarcinoma. These finding are consistent with our study. We found that TAM count and lymphangiogensis is increased with the severity of tumor. We found that TAM count and lymphangiogenesis is increased with the severity of tumor. It is overexpressed in moderately differentiated OSCC than well differentiated OSCC.[30]

Thus a plethora of observation shows that a lymphangiogenesis is associated with chronic inflammination which at a cellular level is regulated by TAM. This tumor included lymphangiogenesis is responsible for lymph node metastasis which is the most important factor responsible for prognosis of the tumor.

Thus we can conclude that TAM count is an significant indicator of tumor biology and the aggressive of the lesion . Immunostainning by CD68 and D@-40 can help in the prediction of tumor behavior and can be suitable target for molecular tumor therapy.

Such a correlation of TAM and lymphangiogensis with regards to lymph node metastasis has not been documented in cases of OSCC .With the help of our study we provide evidence that increased expression of Podoplanin correlates with higher incidence of lymph node metastasis and can be added to the other predictive markers.

The lymphangiogenic characteristics of TAMs have been demonstrated in many tumor models in which blocking macrophage recruitment or depleting macrophages correlated with decreased LVD and suppressed LN metastasis. Tumor VEGF-C levels and blood vessel density were also decreased following this treatment. As tumor lymphangiogenesis is primary cause for lymph node metastasis that promote systemic spread and shorten patient survival. It can thus be of great importance as therapeutic depletion of macrophages can be used as an adjuvant treatment in cases of OSCC.

Macrophages display great plasticity so any one biomarker is insufficient to identify the dynamicity shown by TAMs in a variable and ever-changing tumor microenvironment. Thus, a cocktail of biomarkers along with modern sophisticated and advanced scientific techniques need to be identified which will serve the purpose of identification and quantification if the entire range of the phenotypically different TAMs.

More studies with increased sample size and inclusion of other factors like tumor size, distant metastasis, recurrence and survival of patient can help in establishing TAM count and lymphangiogenesis as an indicator of prognosis in oral cancer.

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