Tumor Associated Macrophages: A Review with Institutional Study

Sreepreeti Champatyray[1], Priyanka Debta[2], Neeta Mohanty[3], Saurjya Ranjan Das[4]

[1]Assistant Professor, [2]Professor, [3]Professor & Head, Department of Oral Pathology and Microbiology, Institute of Dental Sciences, [4]Associate Professor Department of Anatomy, Institute of Medical Sciences & Sum Hospital, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India

Abstract

Aims: To study the expression of tumor-associated macrophages in different grades of squamous cell carcinoma and find the association between their expression and local recurrence, metastasis.

Methodology: Thirty tumor biopsy samples of oral squamous cell carcinoma in T1N0M0 and T2N0M0 stages. Ten biopsy specimen of normal oral mucosa that are taken during minor oral surgical procedure with patient consent.

Result: Macrophages were found to have better specificity for local recurrence, metastasis, and survival. Further, higher expression of biomarkers was associated with shorter survival. We have analyzed the differential expression of MMP 9 in different grades of OSCC & compare their expression with different TNM staging of tumors.

Conclusions: Concluding this review of tumor-associated macrophages in comparison with OSCC cases. Since metastases are the principal cause of death in cancer patients, a greater understanding of the process of tumor invasion and metastasis is essential in leading to the identification of new therapeutic targets.

Keywords: *Immunohistochemistry, OSCC, Tumor-Associated Macrophages.*

Introduction

Oral carcinoma is amongst the leading malignancies worldwide, with squamous cell carcinoma (SCC) as the most common type accounting for over 90% of cases[1]. The high rate of relapse in tumor indicates the inadequacy of current prognostic predictors,that is, histological and clinical assessments, in predicting metastatic potential,as well as the need to investigate additional determinants among oral cancers[2]. In recent years, it has been accepted that to effectively treat cancer, the tumor must be considered as an entity containing both the cancer cells and the surrounding tissue which together forms the tumor microenvironment (TME). In squamous cell carcinoma, the TME may even be dominant over cancer cells[3]. TME contains a mixture of heterotypic cells such as cancer-associated fibroblasts (CAFs), smooth muscle cells, endothelial cells, neutrophils, lymphocytes, and macrophages[4].

Normally macrophages are important cells in wound healing providing aids for tissue cell growth, tissue matrix remodeling, and angiogenesis. There are 2 types of macrophages M1 & M2. M1 macrophages phagocytose & destroy microbes,eliminate cells, present to the antigen to T-cells for an adaptive immune response & produce high levels proinflammatory cytokines &

Corresponding Author:
Dr. Saurjya Ranjan Das
Associate Professor Department of Anatomy, Institute of Medical Sciences & Sum Hospital, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India
e-mail: saurjyadas@gmail.com

helps in tumor suppression. M2 macrophages promote IL-4,IL-10,TGF,PGE2 exposure to TH2 responses, suppresses TH1 mediated inflammation & helps to increase tumor development. Hence Macrophages are described as "double-edged sword" means capable of both promoting & opposing tumor growth[5,6]. Tumor-associated macrophages (TAM) have differentiated into the M2 phenotype which involves angiogenesis, immunosuppression & activation of tumor cells. TAMs release reactive oxygen species, tumor necrosis factor (TNF)-α, Interleukin (IL)-6 and IL-1β, promoting DNA damage, transformation and cancer cell survival.[6]

This tumor-associated macrophages conglomerate with oral squamous cell carcinoma in a metastatic role as a review paper.

Aim: To aim & evaluate the expression of TAM in different grades of OSCC cases.

Objectives:

- Evaluation of expression of TAM infiltration in OSCC.
- Comparison of TAM expression with clinical parameters of OSCC.
- Comparison of TAM with different grades of OSCC.
- Comparison of TAM expression with local metastasis in OSCC cases.
- Correlation of expression of TAM in OSCC cases.

Tumor Associated Macrophages: Macrophages are derived from CD34+ bone marrow progenitors that continually proliferate and shed their progeny into the bloodstream as promonocytes[7]. They then develop into monocytes and extravasate into tissues where they differentiate into a specific type of ''resident'' tissue macrophage[8]. Macrophages also form a major component of the inflammatory infiltrate seen in both primary and secondary tumors.

Broadly macrophages divides into

(a) Classically activated type 1 Macrophages- M1

(b) Alternatively activated type 2 Macrophages- M2

M1 macrophages phagocytize & destroy microbes, eliminate cells, present to the antigen to T cells for an adaptive immune response & produce high levels of pro-inflammatory cytokines. M2 macrophages promote IL-4, IL-10, IL-13, TGF, PGE2 exposure to TH2 responses, suppress TH1 mediated inflammation IL -10. M2 regarded as an increase in tumor development. Thus macrophages described as "double-edged sword" means capable of both promoting & opposing tumor growth[6]. M2 macrophages are called as TAM

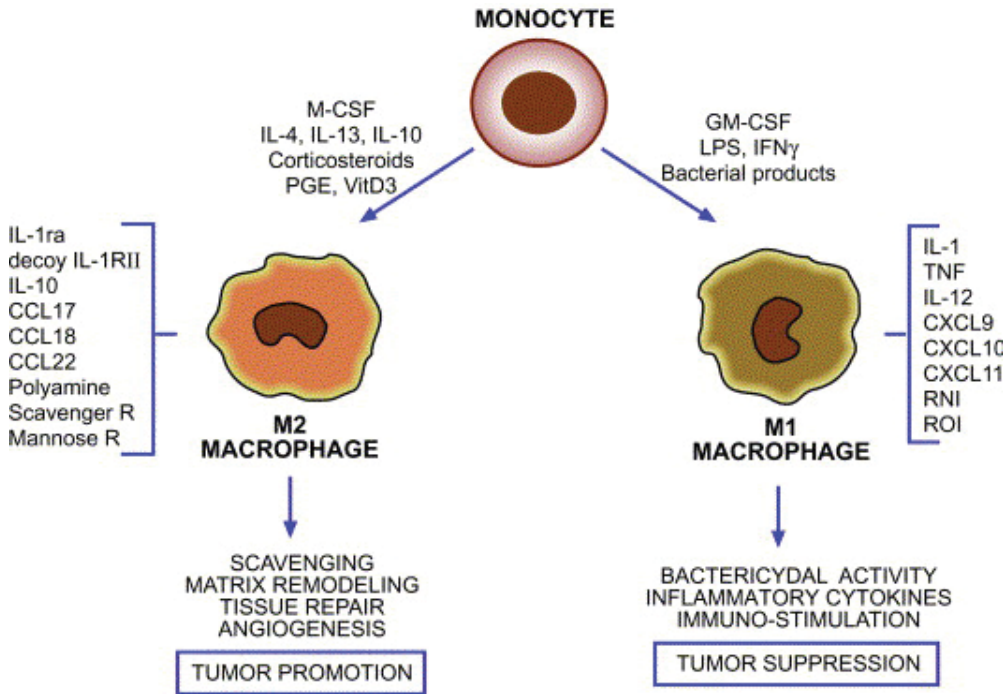


Figure 1: Shows Dual Role of Tumour Associated Macrophages

Roles of TAM: Tumor Invasion: In polyomavirus middle T oncoprotein (PyMT-)induced mammary tumors, macrophages are present in areas of basement membrane breakdown and invasion during the development of early-stage lesions [7]. TAMs secrete a variety of proteases to breakdown the basement membrane around areas of proliferating tumor cells[8]. This finding, together with other results showing the up-regulation of proteolytic enzymes like cathepsin B in macrophages present at the same locations, indicates that TAMs could be involved in the invasion of tumor cells into surrounding normal tissue[8].

Tumor Growth: TAM infiltration positively correlates with tumor cell proliferation as measured by MIB-1 levels in breast carcinomas[14], Ki67 levels in endometrial carcinomas[10], or mitotic index in renal cell carcinoma[9]. This phenomenon is known to involve factors like TGF-h1 being expressed by inflammatory cells in wounded tissues.

Tumor angiogenesis. TAMs release several potent proangiogenic cytokines and growth factors, such as VEGF, TNF-a, IL-8, and bFGF. Additionally, they express a broad array of angiogenesis-modulating enzymes, including MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase- 2 (COX-2).[9] In areas of transient (avascular) and chronic (perinecrotic) tumor hypoxia, macrophages cooperate with tumor cells to induce a vascular supply for the area by up-regulating several angiogenic growth factors and enzymes. These diffuse away from the hypoxic area and together with other pro-angiogenic stimuli in the tumor microenvironment, stimulate endothelial cells in neighboring, vascularized areas to migrate, proliferate, and differentiate into new vessels[2].

Immunosuppression: Macrophages in hypoxic areas secrete factors that suppress the antitumor functions of immune effectors within the tumor. Unlike macrophages from healthy tissues, which are capable of presenting tumor-associated antigens, lysing tumor cells, and stimulating the antitumor functions of T cells and NK cells, TAMs in the tumor microenvironment lack these activities, leaving the host without the ability to mount an effective antitumor immune response[2,6].

Metastasis: A subpopulation of TAMs associated with tumor vessels secretes factors like EGF to guide tumor cells in the stroma toward blood vessels where they then escape into the circulation[7]. In the stromal compartment (both the acellular regions and others where they are in close contact with tumor cells), TAMs secrete growth factors to stimulate tumor cell division and/or undefined factors that promote tumor cell motility[9,10]. TAMs seem to play roles in both the release of metastatic cells from the primary tumor as well as the establishment of secondary tumors at distant sites.

To summarize, analysis of the expression of protumor molecules by TAMs in human tumors and functional studies in macrophage depleted murine tumor models have shown TAMs to play an important part in various key steps in tumor progression. It seems that in early preinvasive lesions, tumor cells release chemokines to attract macrophages and other inflammatory cells into stromal areas surrounding the tumor. Macrophages are also found in dense clusters as part of leukocytic infiltrates in areas of focal breakdown of the basement membrane. Tumor cells are thus able to escape the confines of the basement membranes and migrate across into the stroma of surrounding healthy tissues, where they, together with macrophages, stimulate angiogenesis. These newly formed vessels sprout into the early tumor, providing nutrients and oxygen for tumor growth and supplying multiple exit routes for metastasizing cells[13,14].

Methodology

The present study was done in the Department of Oral & Maxillofacial Pathology & Microbiology, IDS between 2013 to 2016. Approval from the institutional ethics committee of " Siksha O Anusandhan University was obtained to carry out this work.

Clinicopathologic information on each case including age, gender, site of location, habit was collected from the department.

Inclusion Criteria: Patients of all age groups with histopathological diagnosed primary carcinoma cases.

Exclusion Criteria:

- Metastatic tumor in the oral cavity
- Patients having carcinoma with any secondary infections.
- Patients having carcinoma with immuno compromised disease (HIV)
- Tumors which are under treatment of chemotherapy or radiotherapy.

Sample size of the study:

- 30 blocks specimens i.e. 10 well-differentiated, 10 moderately differentiated, 10 poorly differentiated & 10 normal block

Table 1. Scoring criteria for evaluating expression of TAM in our study.

Macrophage Count	Total Score
< 10 %	Score – 0
10-30 %	Score – 1- 2
30- 50 %	Score – 3- 4
>50 %	Score - 5 -6

The results were categorized as negative & positive respectively, based on total score: if total score: 0-2- Negative; 3-6 – Positive. For evaluation of inter/ intra examiner consistency slides are observed by two more examiner for scoring of TAM. The results were categorized as negative & positive respectively based on scoring are:TAM Negative- 13; TAM Positive – 17

Results

The data was collected from individuals cases & organized systemically. Compiling all data was formulated in various tables & graphs were derived from statistical analysis,for easy interpretation of results was done using SPSS software.

Table 2. Comparing the macrophage count with habit

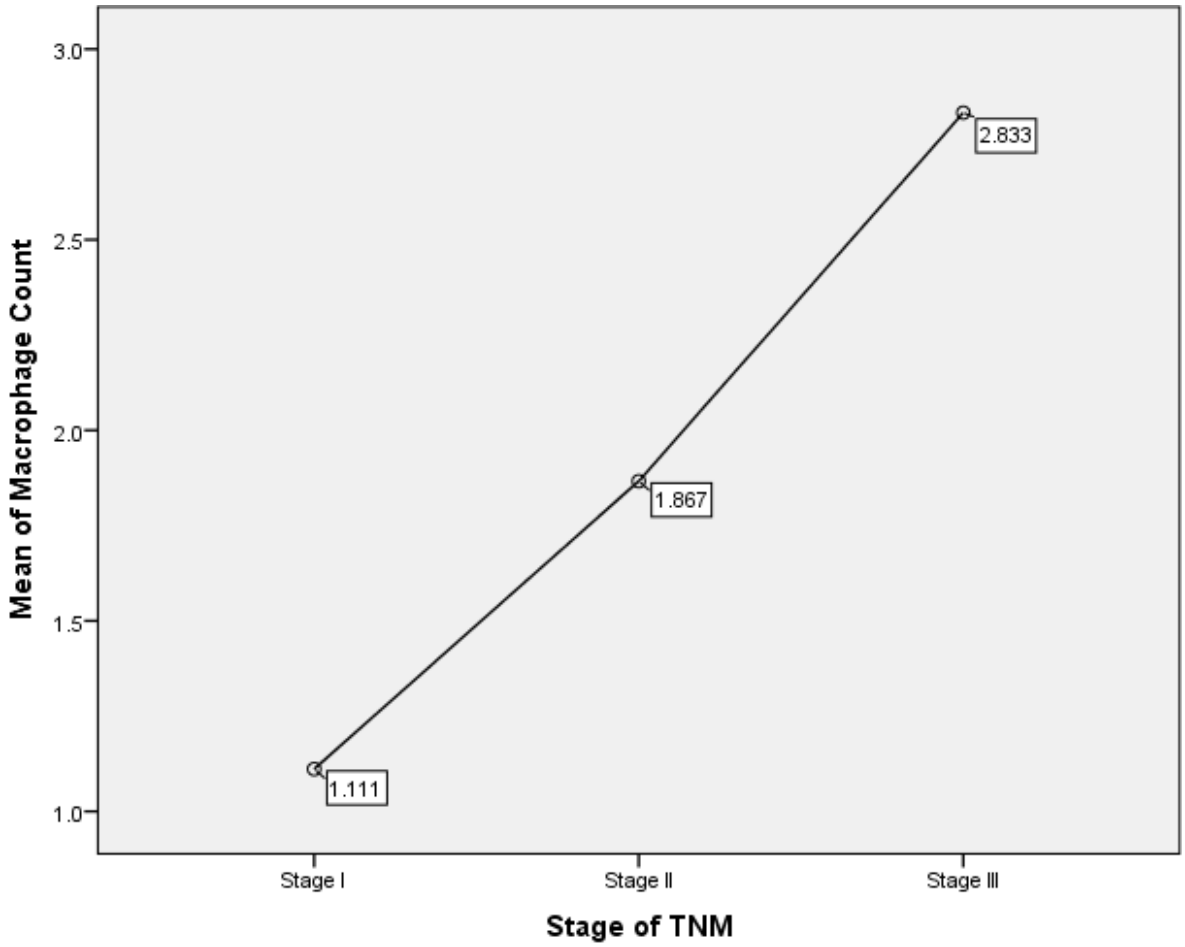
		N	Mean	Std. Deviation	P-value
Macrophage Count	Smoking	8	1.50	.756	0.119
	Chewing	15	1.73	1.033	
	Both	7	2.43	.535	
	Total	**30**	**1.83**	**.913**	

Table 3. Comparison of Stage of TNM with TAM

	N	Mean	Std. Deviation	p-value
Stage I	9	1.11	.782	0.0001
Stage II	15	1.87	.743	
Stage III	6	2.83	.408	
Total	**30**	**1.83**	**.913**	

Table 4. Comparison of expression of TAM between normal group & OSCC group (positive & negative cases)

			mac_rec1		Total	X^2	P-value
			1.00	2.00			
Type of tumor	Normal	Count	10	0	10	9.855	0.002
		% within Type of tumor	100.0%	0.0%	100.0%		
	OSCC	Count	13	17	30		
		% within Type of tumor	43.3%	56.7%	100.0%		
Total		Count	23	17	40		
% within Type of tumor		57.5%	42.5%	100.0%			



Graph 1. Shows Comparision of Stage of TNM with Tumor-Associated Macrophages

Table 5.comparison of expression of TAM between positive & negative cases of OSCC group

	mac_rec1	N	Mean	Std. Deviation	Std. Error Mean	P-value
Macrophage Count	1.00	13	.92	.277	.077	
	2.00	17	2.53	.514	.125	0.0001

Table 6. Tumor-Associated Macrophages with Metastasis using t-test

	Metastasis	N	Mean	Std. Deviation	t	P-value
Macrophage Count	No Metastasis	24	1.58	.830	5.261	0.0001
	Metastasis	6	2.83	.408		

Table 7. Significant relation between TAM in different grades of OSCC group

		N	Mean	Std. Deviation	P-value
Macrophage Count	Normal	10	.50	.527	0.0001
	Well	10	1.40	.843	
	Moderate	10	1.80	.919	
	Poor	10	2.30	.823	
	Total	40	1.50	1.013	
	Total	40	1.63	1.125	

Observation: Table 2 represents the comparison between TAM count with a habit of OSCCcases which is not significant. Table 3 represents the Comparision of the Stage of TNM with TAM is statistically significant. ($p < 0.05$). Table 4 represents a comparison of expression of TAM between normal group & OSCC group positive & negative cases which is statistically not significant ($p > 0.002$). Table 5 represents the comparison of expression of TAM between positive & negative cases of OSCC group shows statistically significant. Table 6TAM with Metastasis using t-test shows statistically significant. Table 7 Significant relation between TAM in different grades of OSCC group shows statistically significant. Graphrepresents Comparision of Stage of TNM with TAM is statistically significant with mean values (stage I- 1.111, stage II- 1.867, stage III-2.833) ($p < 0.05$).

Discussion

Squamous cell carcinoma (SCC) is considered a major problem worldwide due to its extremely high prevalence (90%) among oral cancers[2,6]. The high rate of relapse in this tumor indicates the inadequacy of current prognostic predictors, that is, histological and clinical assessments, in predicting metastatic potential[2]. So there is a need to investigate additional determinants. So in this study, we have investigated macrophages infiltration in different grades of OSCC cases. So in our study, we evaluated the expression of macrophages in different grades of OSCC cases. There are many method to evaluate MMP 9 like Gelatin Zymography, Immunofluorescence (IFC), Gel Electrophoresis, PCR, IHC, Western Blot Assay, ELISA.

There are various method used to evaluate macrophages are immunofluorescence (IFC), Immunohistochemistry, flow cytometry, qRT-PCR. & routine H & E stain[11,12] In our study we have used H & E (Hematoxylin & Eosin) stain to evaluate TAM as cancer-associated inflammation includes the so-called tumor-associated macrophages (TAMs) which are generally thought to resemble M2-type macrophages and we can see the presence of macrophage with our routine H & E stain.[12,13]

For Macrophages: scoring criteria < 10% is scored as 0, 10-30 % is scored as 1-2, 30-50 % scored as 3-4, > 50 % is scored as 5-6.

OSCC is the most common malignant neoplasm. It may occur at any intraoral site & generally involve tobacco users in the middle age group. Higher incidence of OSCC seen frequently in males[15]. In our study we found 80 % of OSCC cases below 60 yrs of age, 60 %were males & 40 % were females; majority cases (53.33%) had a lesion in the buccal mucosa. According to Taiming Dai et al[15,17], there was a significant difference in comparison of MMP-9 expression between men and women, suggesting there might be a higher potential for OSCC invasion in male patients.

In our study, we have compared smoking & non-smoking form of tobacco with the infiltration of TAM. We found that TAM infiltration is not significantly associated with different forms of tobacco habit pattern [13]. In our study mean score of both TAM expression increases from stage 1- stage III, and it was statistically significant ($p < 0.05$) which suggest that MMP 9 expression is directly proportional to clinical stages of tumor & this finding is in concordance with a study done by Taiming Dai et al [15], where as in other studies like Aparnat et al [9], Elahi et al[68] they have not found any correlation between TAM & MMP 9 expression with different stages of TNM. There was a statistically significant infiltration of TAM in OSCC cases as compared to the normal groups. Total no. of OSCC cases were divided into positive & negative groups according to the expression of TAM. We have found MMP 9 expression 23 positive cases & 7 negative cases & TAM expression 17 positive cases & 13 negative cases. The difference between TAM expression in positive & negative cases was found statistically significant.[8] We also found that there was a statistically significant correlation between the infiltration of TAM in OSCC cases[15,17]. We also found that there was an increase expression of TAM with different histological grades of oral squamous cell carcinoma: TAM in Well-differentiated 1.40+_.843, mod differentiated 1.80+-.919,to poorly differentiated 2.30+-.823 (lower to higher mean & s.d value) . Thus their expression is increasing from well to poorly differentiated squamous cell carcinoma. But the difference between the expression of TAM in different grades of OSCC was not statistically significant[15] found that positive correlation between the macrophage count. They also suggested that macrophage in OSCC, which jointly promote the invasion and metastasis of the tumor.

The positive expression rate was much higher in OSCC in comparison to normal mucosa and it had a close correlation with TNM staging of the tumor and condition of local metastasis TAM over-expressed in many other malignant tumors like breast, prostate, liver,

lung & bladder cancers their expression was correlated to poor prognosis[9,10,11,12]. We have compared TAM expression in OSCC cases with local metastasis which is found statistically significant ($p < 0.05$) which in concordance with the other studies done by Daiming Tai et al [16]. Thus it is proved that invasion & metastasis of tumors are regulated by quantities of factors in a cellular mixture of OSCC[4]. It may also be helpful to evaluate local metastasis in OSCC cases. Since metastases are the principal cause of death in cancer patients, a greater understanding of the process of tumor invasion and metastasis is essential in leading to the identification of new therapeutic targets.

In this study, we have used H & E stain instead of IHC for macrophages identification due to lack of funds, but the expression of macrophages can be better evaluated by using specific IHC markers like CD 68, CD 13. In this study, although special care has been taken to avoid false-positive results,but IHC is very technique sensitive. Thus further studies should be done with a larger sample size along with the use of other specific method to confirm the expression of both TAM in OSCC cases & their expression should be correlated with the IHC technique.[17]

Conclusion

Concluding this review of tumor-associated macrophages in comparison with OSCC cases. Since metastases are the principal cause of death in cancer patients, a greater understanding of the process of tumor invasion and metastasis is essential in leading to the identification of new therapeutic targets. In this study, we have used the H & E stain instead of IHC for macrophages identification due to a lack of funds. In this study, although special care has been taken to avoid false-positive results,but IHC is very technique sensitive. Thus further studies should be done with a larger sample size along with the use of other specific method to confirm the expression of both TAM in OSCC cases & their expression should be correlated with the IHC technique.

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