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The expression of CD68+ Macrophages in Oral Squamous Cell Carcinoma a Clinicopathologic Correlation

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Abstract:

Context: The most common malignancy of oral cavity is the squamous cell carcinoma (OSCC). Macrophages function in wound healing, helping in cell growth, angiogenesis and tissue matrix remodelling and have a role in tissue repair, development, homeostasis and immunity. Tumour-associated macrophages (TAMs) have been suggested to function in tumour cell proliferation, tumour cell migration & invasion, and tumour angiogenesis. The cells of monocyte-macrophage lineage in normal and pathologic conditions can be identified by CD68.

Aims: To quantify the immunohistochemical expression of CD68 in Oral squamous cell carcinoma and correlate it with different grades of OSCC and TNM staging

Settings and Design: A cross-sectional Observational study

Methods and Material: The study population comprised of biopsies obtained from 40 cases of OSCC graded according to Bryne's grading and 20 cases of normal oral mucosa. 5µm thick sections were stained immunohistochemically using monoclonal mouse antihuman antibody CD68. Mean number of CD 68 positive stained cells were counted.

Statistical analysis used: Statistical tests used in the study were Students unpaired t test, One way ANOVA, Chi-square Test, Neuman-Keuls Multiple Comparison Test, Multiple Logistic Regression Analysis

Results: Using student's unpaired t test, a statistically significant difference was observed in mean CD68 count in the Control Group (31.38 ± 26.60) and the OSCC Group (200.53 ± 74.13). One way ANOVA test showed statistically significant difference in CD68 count in the three histological grades of OSCC (132.60 ± 34.45, 222.16 ± 40.03 and 293.16 ± 56.75 in Grade I, II & III respectively). One way ANOVA test showed a statistically significant difference in CD68 count in all TNM stages of OSCC patients.

Conclusions: TAMs were heterogeneously detected in OSCC and their presence was higher in tumours with advanced grade of OSCC and TNM stage. TAMs are associated with creation of a permissive environment for tumour invasion in OSCC.

Key-words: Oral Cancer, Oral Squamous cell carcinoma, Carcinogenesis, Macrophages, Tumour-associated macrophages, CD68

Introduction:

Rudolf Virchow in 1863 demonstrated inflammatory leukocytes in tumors. Solid tumours comprise not only tumour cells, but also the tissue matrix and various stromal cells.[1] One such group of stromal cells are **macrophages**, at times called **macrophagocytes**Monocytes differentiate into macrophages in tissues. Russian bacteriologistMechnikov discovered macrophages in 1884.[2] Macrophages function in wound healing, providing aids for tissue cell growth, tissue matrix remodelling and angiogenesis. The macrophages are seen in all tissues and have variety of functions. They act in homeostasis, tissue repair and immunity. Macrophages, are prehistoric cells in Metazoan phylogeny. They are arranged in specific patterns in tissues.

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Macrophages can be classified under mononuclear phagocytic system, that includes them as well as their bone marrow progenitors. The adult macrophages are the terminal cells of the mononuclear phagocytic lineage while the tissue macrophages are derived from peripheral monocytes. [2]
It has been known that the tissue macrophages maintain homeostasis by responding to external agents and physiological changes. Many a times, the homeostatic and reparative functions are weakened by repeated damages, changing the role of macrophages to being helpful to disease states specially the malignant tumours.[2]

The cells of monocyte-macrophage lineage in normal and pathologic conditions can be identified by CD68 immunologically and microphages have been suggested to function in tumour cell proliferation, migration & invasion, and tumour angiogenesis.[3, 4]Hence, in this study we tried to explore the expression of these cells and check if there is any correlation with different grades of OSCC.

Aim: To quantify and study the expression of CD68 in OSCC using immunohistochemistry.
Objectives: To evaluate the different grades of OSCC histopathologically.
To evaluate, quantify and correlate the expression of CD68 in different grades of OSCC.

Subjects and Methods:
A cross-sectional study was conducted, comprising of 40 cases of OSCC and 20 cases of normal oral mucosa. Informed written consent was obtained from enrolled patients. This study was based on biopsy specimens that were histopathologically diagnosed as positive for OSCC & graded as Grade I OSCC (16 cases), Grade II OSCC (16 cases), Grade III OSCC (8 cases) and were obtained from the Department of Oral Pathology and Microbiology, SwargiyaDadasahebKalmeghSmruti Dental College & Hospital, Nagpur, Maharashtra, India. Gingival or vestibular normal oral mucosa was obtained from patients undergoing extractions (without any signs of gingival or periodontal inflammation). Tissue specimens diagnosed as positive for OSCC were graded histopathologically as per Bryne's grading system.[5]The other slides were subjected to immunohistochemical analysis. Five micron thick paraffin sections taken on lysine-coated slides were stained immunohistochemically using monoclonal mouse antihuman antibody CD68. The number of CD68 positive cells was counted under high power magnification. Evaluation was done as per Bryne's grading system.
Inclusion criteria included incisional & excisional biopsies of primary oral squamous cell carcinoma with and without lymph node metastasis. Normal oral mucosa from patients undergoing extractions, gingivoplasty or crown-lengthening procedures served as control tissue. Exclusion criteria for Control group included, biopsy of the subjects with signs of inflammatory gingival or periodontal disease while for the study group, the exclusion criteria included benign neoplasms arising from oral mucosa, primary malignancies and metastatic tumours of oral cavity and jaw bones other than OSCC.

Equipment and materials used for staining were monoclonal mouse anti-human CD68 antibody, Clone PG-M1, 6ml, Code No.- M 0876 (DAKO) & Super Sensitive TM Polymer- HRP IHC Detection System HRP / DAB. Catalogue No. - QD400-60KE, from BioGenex Consolidated staining system— San Ramon, CA 94583, USA.

1. ***Sectioning:*** From each paraffin embedded block, one section was cut by using rotary microtome (Leica R.M.2135, The sections were standardized by maintaining the thickness at 5 μm.
2. ***Fixing*:** The slides were fixed overnight at 60°C in the incubator.
3. ***Clearing:*** The sections were cleared by passing them through 3 changes of xylene for 5 minutes each.
4. ***Rehydration:*** The sections were rehydrated by passing them through absolute alcohol, 90% alcohol and 70% alcohol for 5 minutes each and then the slides were kept immersed in distilled water for 5 minutes. 5. ***Buffer preparation:*** The required amount of the reagents was accurately measured by using a digital balance.

Presence of brown coloured precipitate at the site of target antigens was indicative of positive immunoreactivity. The overall slide background was clear without any extraneous deposits. Tissue sections

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of highly inflamed pericoronal tissue were taken as the positive control in our study. The evaluation of the study cases was done subsequently in a similar way. Stromal cells positive for Cytoplasmic immunostaining for CD68 were considered as macrophages. CD68 positive cells, excluding those in areas of inflammation were counted.

CD68 positive macrophages were assessed by selecting 5 Hot-spots (most dense fields) showing in the connective tissue below the epithelium for both normal oral mucosa and OSCC. Positively stained cells were counted in at least five Hot spots at x400 magnification using Image J image analysis software. Three high power fields from the five hotspots showing highest counts were selected and their mean value was calculated. The results were presented as the mean number of CD68 positive cells per high power field. (Fig. 1) In order to eliminate the observer bias, the count was performed by three different observers with good inter-observer agreement.

The data was subjected to statistical analysis using SPSS 17.0 and Graph Pad Prism 5.0 version and $p<0.05$ at Confidence Interval (CI) of 95%, was considered as level of significance. Statistical tests used in the study were Students unpaired t test, One way ANOVA, Chi-square Test, Neuman-Keuls Multiple Comparison Test, Multiple Logistic Regression Analysis.

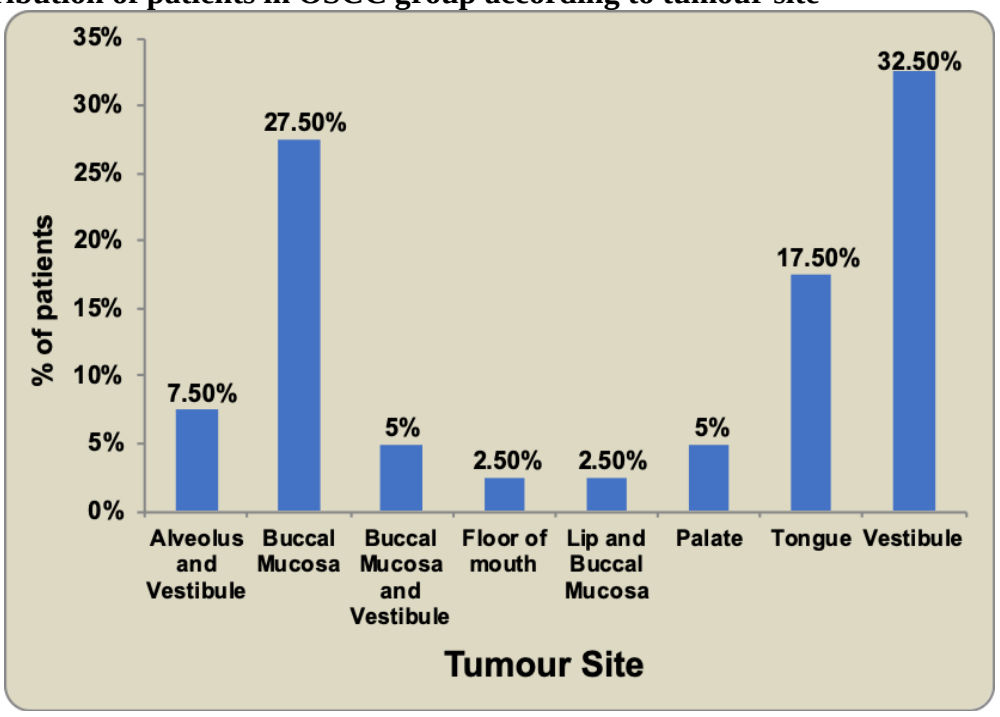
Results:

A total of 40 sections of previously histologically diagnosed, paraffin embedded tissue blocks of OSCC and 20 sections of normal oral mucosa were assessed for the expression of CD68 which is a marker for tumour-associated macrophages. Normal oral mucosa was taken as negative control and inflammatory oral mucosa served as the positive control in all cases for the specificity of the stain.

In our study, we observed that 39 out of 40 cases were tobacco consumers, either smoked or smokeless forms. A total of 97.5% of the patients of OSCC groups were associated with adverse habits and only 2.5% (1 case) reported as not having any adverse habit, but showed the presence of sharp cusps, thus proving the different forms of tobacco to be playing a significant role in the development of OSCC.

The most common site of occurrence was observed to be the buccal vestibule, buccal mucosa, lateral border of tongue, alveolus, floor of mouth and labial mucosa. This can be attributed to the site of quid placement, usually being the buccal vestibule (Graph 1).

Graph 1: Distribution of patients in OSCC group according to tumour site



There were 16 (40%) cases each of Grade I (Figure 2) and Grade II OSCC (Figure 3) and 8 cases belonged to Grade III (Figure 4) category (Graph 2).

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Figure 1-**Cell counter window of Image J image analysis software**

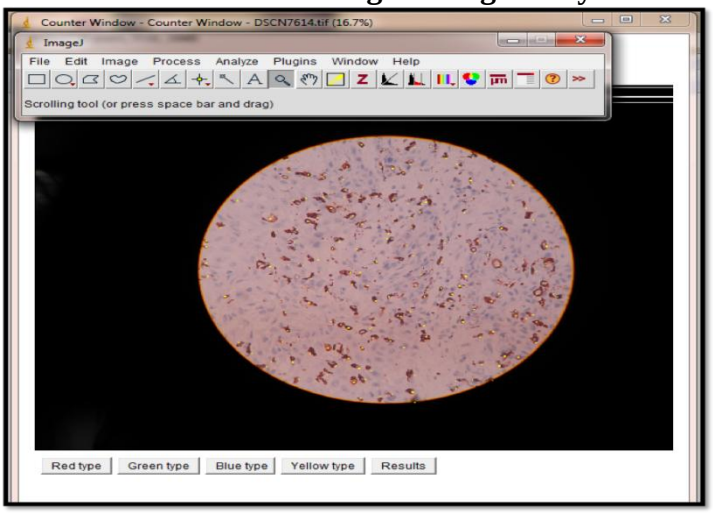

Figure 2- CD68 positive cells counted in Grade I OSCC at 40X magnification using Image J image analysis software

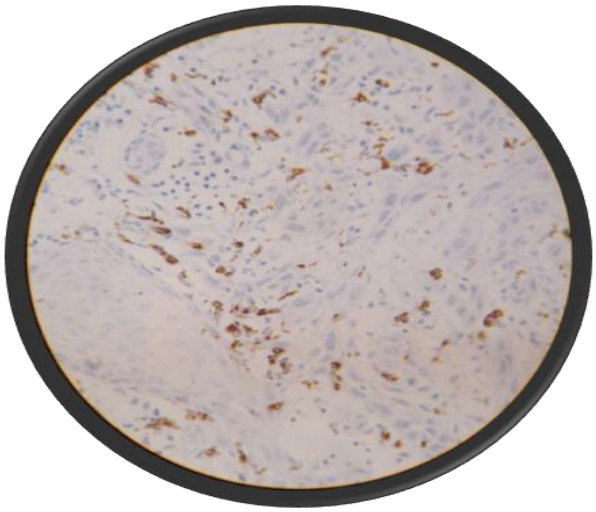

Figure 3- CD68 positive cells counted in Grade II OSCC at 40X magnification using Image J image analysis software

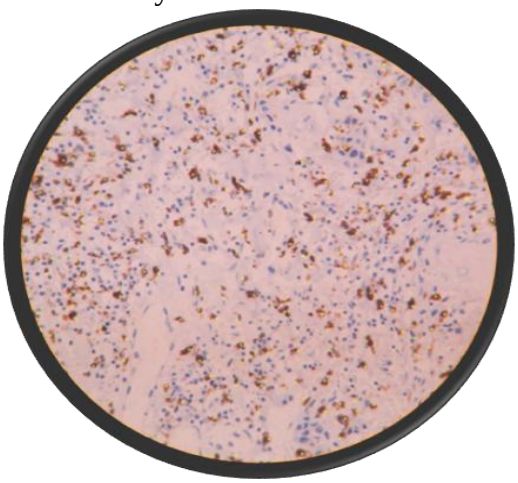

Figure 4- CD68 positive cells counted in Grade III OSCC at 40X magnification using Image J image analysis software

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 5552- 5561
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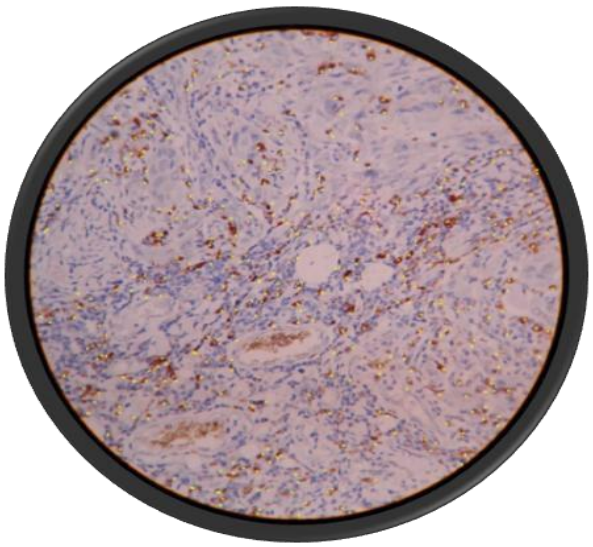
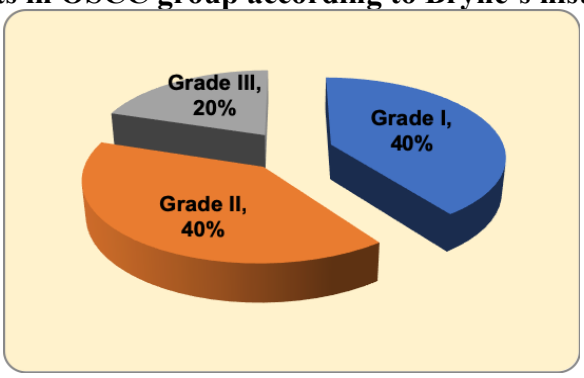
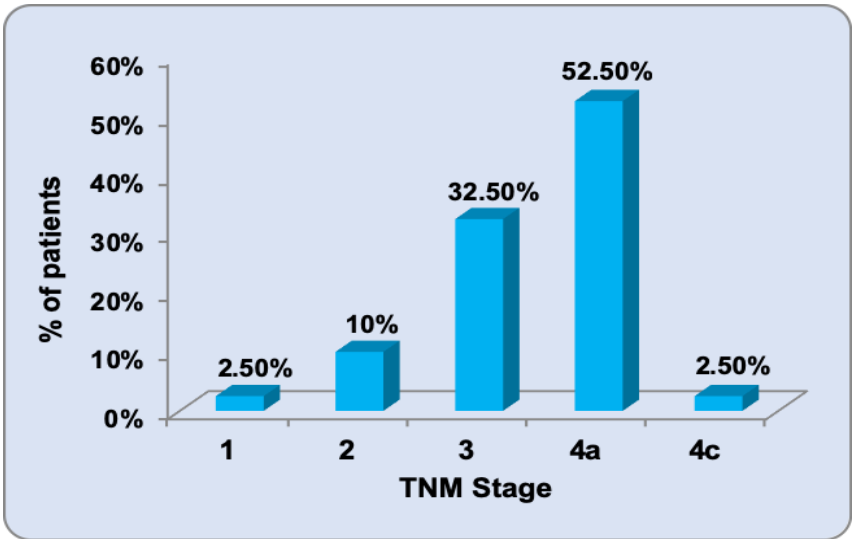


Graph 2: Distribution of patients in OSCC group according to Bryne's histological tumour grading



TNM staging was done during history taking and clinical examination for each case. We observed that 2.5% of the patients belonged to Stage 1 and Stage 4c, each, as per AJCC TNM Staging, 10% belonged to Stage 2, 32.5% belonged to Stage 3 and 52.5% of them belonged to Stage 4a respectively (Graph 3). The data was subjected to statistical analysis with unpaired t test, One way ANOVA, Chi-square test, Neuman-Keuls Multiple Comparison Test and Multiple Logistic Regression Analysis.

Graph 3: Distribution of patients in OSCC group according to TNM staging



Mean CD68 count in patients of OSCC was 200.53 ± 74.13 and in Control group it was 31.38 ± 26.60. Using student's unpaired t test statistically significant difference was observed in mean CD68 count in both the groups (t=9.85, p-value=0.000 at CI=95%) (Table 1).

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Table 1: Comparison of CD68 count in OSCC and Control groups

Group	N	Mean	Std. Deviation	Difference	t-value	p-value
OSCC	40	200.53	74.13	169.15±17.16	9.85	0.000 S, p<0.05
Control	20	31.38	26.60			

N: Number of cases; S: Significant

Mean CD 68 count for the patients with Grade I OSCC was 132.60 ± 34.45, for Grade II OSCC patients it was 222.16 ± 40.03 and for Grade III OSCC patients it was 293.16 ± 56.75. By using one way ANOVA statistically significant variation was found in CD68 count in the three histological groups (F=43.08, p-value=0.000 at CI=95%) (Table 2).

Table 2: Correlation of Bryne's histological grading with CD68 count

Histological Grading	N	Mean	Std. Deviation	Std. Error	F-value	p-value
Grade I	16	132.60	34.45	8.61	43.08	0.000 S, p<0.05
Grade II	16	222.16	40.03	10.00		
Grade III	8	293.16	56.75	20.06		

N: Number of cases; S: Significant

By using Newman-Keuls multiple comparison test, statistically significant difference was observed between Grade I and Grade II OSCC (p=0.000), between Grade I and Grade III OSCC (p=0.000) and between Grade II and Grade III OSCC (p=0.001), all at CI=95% respectively (Table 3)

Table 3: Newman-Keuls multiple-comparison test

Grading of OSCC		Mean Difference (I-J)	Std. Error	p-value
Grade I	**Grade II**	89.56*	14.74	0.000 S, p<0.05
Grade I	**Grade III**	160.56*	18.06	0.000 S, p<0.05
Grade II	**Grade III**	71.00*	18.06	0.001 S, p<0.05

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S: Significant

Mean CD 68 count in patients with TNM stage 1 was 141.33, in TNM stage 2, it was 112.83 ± 45, in TNM stage 3, 188.66 ± 64.17, in Stage 4a it was 225.36 ± 73.46 and in TNM stage 4c it was 243.66. One way ANOVA test showed a statistically significant variation in CD68 count in all TNM stages of OSCC patients (F=2.72, p-value=0.045 at CI=95%) (Table 4)

Table 4: Correlation of TNM staging with CD68 count

TNM staging	N	Mean	Std. Deviation	Std. Error	F-value	p-value
1	1	141.33	.	.	2.72	0.045 S, p<0.05
2	4	112.83	45.00	22.50		
3	13	188.66	64.17	17.79		
4a	21	225.36	73.46	16.03		
4c	1	243.66	.	.		

N: Number of cases; S: Significant

Results of Multiple Logistic Regression analysis showed that the grades of OSCC significantly correlated with CD 68 count i.e., increase in CD68 counts were observed in advancing grades of OSCC. However, other parameters like age, sex and habit did not significantly correlate with CD68 count, as shown in Table 5.

Table 5: Multiple Logistic Regression Analysis

Parameters	Unstandardized Coefficients		Standardized Coefficients	t	p-value
	B	Std. Error	Beta		
CD 68 count	20.76	77.61	-	0.26	0.791 NS, p>0.05
Age	0.91	0.61	0.144	1.48	0.146 NS, p>0.05
Gender	3.38	14.75	0.021	0.23	0.820 NS, p>0.05
Habit	-4.82	50.22	-0.010	0.09	0.924 NS, p>0.05

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Grading of OSCC	79.83	11.04	0.816	7.23	0.000 S, p<0.05
TNM Staging	-3.36	11.60	-0.037	0.290	0.774 NS, p>0.05

S: Significant; NS: Not significant

Discussion:

Squamous cell carcinoma is the commonest of oral cancer that is accompanied by high mortality rate. In normal mucosa, the stromal reaction is triggered by changes in the epithelium. Many causative factors are responsible for changes in oral epithelium from normal to dysplastic to malignant. Many cases of oral dysplasias acquire malignant features and infiltrate the connective tissue.[6]Despite their anatomic & functional differences, the tissue macrophages function in tissue homeostasis. Nonetheless, the reparative and homeostatic properties of these cells can be disrupted by repeated insults, leading to macrophages being responsible in disease states.[2]Tumours are abundantly populated by macrophages. The macrophages are generally believed to participate in anti-tumour response, studies have indicated that many a times they support tumour initiation, their progression and ultimately metastasis.[7] Depending on the expression of cytokines, receptors and effector molecules, the macrophages can be divided into two phenotypes- the M1 and M2. According to M1/M2 paradigm, the macrophages can switch their phenotype from pro-inflammatory M1 to anti-inflammatory M2 and vice versa. Macrophages secrete various cytokines e.g. IFN-γ, TNF-α and IL-6 in presence of chronic irritation or infection that leads to accumulation of other immune cells. This process might lead to tumour initiation. Once tumours become established they cause differentiation so that the tumour-associated macrophages (TAMs) change from an immunologically active state M1 to adopt a trophic immunosuppressive M2 phenotype that promotes tumour progression and malignancy.[7]

Macrophages have been reported to constitute up to 80% of the tissue mass in breast carcinomas. The monocytes reaching into malignant tumours differentiate into TAMs. The levels of chemokines have been reported to be increased along with the numbers of TAMs in tumours. TAMs may increase in hypoxic or necrotic areas of endometrial, breast, prostate and ovarian carcinoma due to the release of macrophage chemo-attractants like EMAP-II, endothelin 2 and VEGF. Macrophages being phagocytic get attracted to hypoxic, peri-necrotic areas along necrotic debris coming out of degenerating cells. TAMs get entrapped due to decreased mobility in presence of hypoxia due to increased expression of the enzyme mitogen activated protein kinase phosphatase (MKP-1) by macrophages.[8]Since CD68 is the best established generic macrophage marker, we evaluated the expression of CD68 in different histological grades of OSCC.[9] In the present study, we observed that the mean CD68 count in OSCC was much higher than the corresponding value in normal oral mucosa. We observed that the TAM count in the OSCC group to be in the range of 25 to 389 cells/mm^{2,} with a mean of **200.53 ± 74.13** cells/mm^2 which gave a statistically significant result when compared with Control Group (**31.38 ± 26.60** cells/mm^2) as well as with TNM stage. ($p<0.05$)

We also observed that the CD68 count increased with advancing grades of OSCC. **Lu CF et al (2010)** measured the macrophages per high-power field in oral squamous cell carcinoma and found significantly increased count in OSCCs with larger tumour size, positive lymph node metastasis, more advanced clinical stages, or recurrence.[10] Similarly, in our study, we correlated CD68 count in OSCC with the TNM stage for each case and found a statistically significant difference (Table 4).

CD68+ count showed a statistically significant variation on comparison with TNM stage ($p=0.045$ at CI of 95%). One case of Grade III OSCC showed metastatic deposits in neck, and was therefore classified as Stage 4c using TNM staging. We found that this case showed the highest CD68 count amongst all TNM stages, having a mean value of 243.66 cells/mm^2 (Table 4). Thus, the high TAM counts are one of the significant indicators which can be used to predict metastasis in OSCC.

Macrophage density was observed to be higher in the invasive Grade III carcinomas ($p<0.05$), compared with Grade I and Grade II OSCC, suggesting that macrophages may be a predictor of tumour aggressiveness (Table 2, 3).

Targeting TAMs by reducing their density in tumour tissues has been proved to effectively alter the tumour microenvironment and suppress tumour growth and metastasis, serving as an efficient therapeutic module. **Luo et al**, in **2006**, demonstrated that legumain, from the asparaginylendopeptidase family that serves as a stress protein, overexpressed by TAMs, acts as an ideal target molecule for vaccine. The authors tried a legumain-based DNA vaccine that worked in murine models in survival of vaccinated mice. The results of our study showed an increase in the number of CD68 positive macrophages which is indicative of their role in tumor invasion and metastasis. It is presumed that the phenotypic role reversal is responsible for higher invasiveness and metastasis.[11]

Though the case selection was done with utmost care fulfilling the inclusion and exclusion criteria, following are the limitations of the study. Intratumoral heterogeneity is a known fact, which could lead to differences in the levels of macrophages in different grades. A future study may be conducted with standardization of cases on basis of histopathology.

The oral potentially malignant disorders like leukoplakia, Oral submucous fibrosis, erythroplakia, lichen planus etc. have been known to transform into malignancy. It would have been interesting to include such cases for comparison of macrophage counts in them with that of oral cancer, however, due to lack of resources and financial constraints, they could not be included. The oral submucous fibrosis is known to induce fibrosis in the oral cavity and the evaluation of macrophage count in such cases could have helped strengthening the postulated role of macrophages in tumour progression. Hence, a future study could be done with inclusions of potentially malignant disorders for quantification of macrophages.

The present study did not include any follow up for the oral cancer cases due to time constraints. Hence, the survival status and the post-treatment levels of macrophages could not be evaluated in this study.

Additional staining with CD163 would have helped to differentiate M1 from M2 macrophages with greater specificity. Use of CD31 would aid in the correlation of TAMs and angiogenesis in tumours.

Conclusion:

Tumour microenvironment is a significant contributor to tumour progression. Macrophages, activated fibroblasts, endothelial cells, inflammatory cells and other extra cellular matrix components form a part of this microenvironment. Most of the tumour-associated macrophages are CD68 positive and represent the majority of tumour stromal cells.

Transdifferentiation of macrophages to TAMs and the associated phenotypic role reversal is crucial and could be one of the early events in tumour progression. The present study was an attempt to reinforce the hypothesis that TAMs are essentially a part of reactive tumour stroma and also to establish the quantitative and qualitative relationship of TAMs in different grades of OSCC. TAMs were heterogeneously detected in OSCC and their presence was higher in tumours with advanced grade of OSCC as well as TNM stage. These findings suggest that TAMs are associated with creation of a permissive environment for tumour invasion in OSCC. It is increasingly clear that the tumour stroma is important in carcinogenesis. Understanding the role of the TAMs will enable us to identify more precise diagnostic modalities, prognostic markers and define novel therapeutic targets.

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