Original Article

Evaluation of density of tumor-associated macrophages using CD163 in histological grades of oral squamous cell carcinoma, an immunohistochemical study

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Abstract

Background: Macrophages account for 30%–50% of the total inflammatory cell population of "tumor microenvironment" that plays an important role in cancer metastasis. M2 macrophages are designated as tumor-associated macrophages (TAMs). They are known to orchestrate all the stages of tumor progression. CD163 is TAMs–M2-specific marker.

Aims: The aim of the study was to evaluate the role of TAMs using CD163 in different histological grades of oral squamous cell carcinoma (OSCC).

Setting and Design: Expression of CD 163 was investigated in 30 histopthologically diagnosed cases of OSCC.

Materials and Methods: Two sections of 4-μ thickness were stained with hematoxylin and eosin, CD163 (Cell Marque, USA). The expression of TAMs with CD163-positive cells was done by counting the number of macrophages in three high-power fields (×400), and the mean number of macrophages per HPF was evaluated.

Statistical Analysis: The statistical analysis was performed using Statistical Software SPSS version 20.0.

Results: CD163 TAMs score increasing in higher tumor, node, metastasis stages with significant positive correlation.

Conclusion: With higher histological grades, CD163 TAMs score increased. Thus, TAMs may be considered as an independent factor for determining the progression of the tumor. The immunotherapeutic approaches to control M2 TAM numbers could protect against progression to malignancy.

Key words: CD163, tumor-associated macrophages, Epithelial mesenchymal transition(EMT), tumor microenvironment

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INTRODUCTION

Cancer is one disease that fits the paradigm that "more we know, less we understand its intricacies."[1] Oral squamous cell carcinoma (OSCC) accounts for nearly 91% of all oral malignancies.[2,3] It is the second-most frequent form and the cause of death from cancer among males in South-East Asia.[4] Approximately 90% of cancer-related death is caused by metastasis.[5] Despite advances in the surgical, chemotherapy and radiotherapy treatment options, the 5-year survival rate has not been improved significantly in the past few years.[6]

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Recent cancer research has made it increasingly clear that tumors are more than insular masses of proliferating cancer cells. The tumor microenvironment, which is formed by multiple cellular and molecular interactions, plays an important role in mediating the biological behaviors of cancer, including OSCC.[7] During the ensuing decade, this notion has been solidified and extended, revealing that the biology of tumors can no longer be understood simply by enumerating the traits of the cancer cells but instead must encompass the contributions of the "tumor microenvironment" to tumorigenesis.[2,8-10] This led Mantovani *et al.* (2009) to propose "tumor inflammation" as "the seventh hallmark of cancer" thereby highlighting the importance of the host environment in disease progression.[11,12]

Macrophages account for 30%–50% of the total inflammatory cell population in the tumor microenvironment.[6,9,13,14] They constitute an extremely heterogeneous population, categorized as M1 (classically activated) and M2 (alternatively activated) phenotype.[15] M2 macrophages are designated as tumor-associated macrophages (TAMs). They are known to orchestrate all the stages of tumor progression,[15,16] neoangiogenesis, metastasis[17] and immunosuppression. CD 163 is TAMs–M2-specific marker.[18]

Recent studies demonstrate that cancer cells undergoing EMT at the invasive front of tumor tissue can establish a suitable microenvironment for tumor progression, where TAMs are always found.[6] However, the interaction between the TAMs and cancer cells undergoing EMT in the OSCC progression remains unknown.

Thus, the aim of the study is the density of CD163-positive TAMs in different histological grades and to investigate the interaction between CD163-positive TAMs and cancer cells in OSCC progression.

MATERIALS AND METHODS

The study group comprised 30 histopathologically diagnosed cases of OSCC with various grades of differentiation according to the AJCC Tumor, Node, Metastasis (TNM) system.[19]

Two sections, each of 4-μ thickness from formalin-fixed paraffin-embedded tissues were stained with hematoxylin and eosin and primary antibody against CD163 (Cell Marque, USA). Immunostaining with CD163 was performed using Sensitive Polymer DAB Detection Kit., (NovoLink Polymer Detection System, Novocastra) [Figure 1-6]. Inflamed tissue used as a positive control for CD163. Five normal mucosa samples are subjected to H and E staining and immunostaining with CD163.

Density of tumor-associated macrophages by CD163[20]

Each section was screened at low power magnification to identify the areas with the highest macrophage density by positive (brown) staining by chromogen. CD163 revealed strong cytoplasmic and membranous staining of M2 macrophages. The expression of TAMs with CD163-positive cells was done by counting the number of macrophages in three high power fields (×400), and the mean number of macrophages per HPF was evaluated. The scoring is done as Score 1 −<50 and Score 2 −>50.

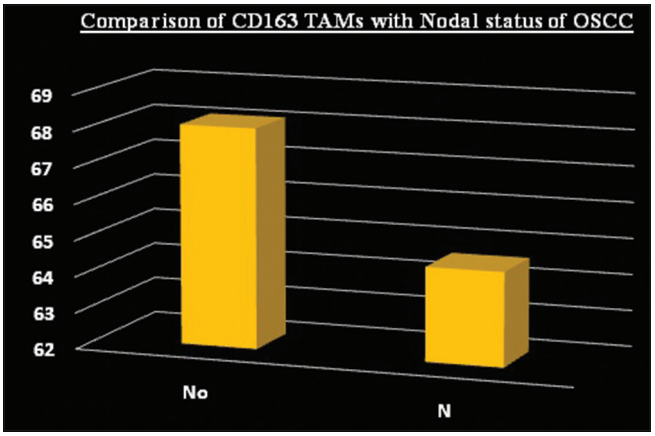
The statistical analysis was performed using Statistical Software SPSS version 20.0. Descriptive statistics were used for demographic data and summarized as mean with standard deviation and as the number with percentage for discrete variables. Kruskal–Wallis ANOVA and pairwise Mann–Whitney U-tests were applied to evaluate the significant differences among the mean values in different groups.

Results with "$P < 0.05$" were considered to be statistically significant at 95% confidence interval.

RESULTS

On analysis of the demographic data, the majority of patients belonged to the age group of 40–60 years, with the mean age at 48.5 years with predominantly male (90%). The most predominant site for OSCC was found to be of the gingivobuccal sulcus (56.67%), then tongue (33.33%) and (10%) the alveolus.

On TNM staging, the majority of the cases belonged to Stage II 17 (56.67%), followed by 12 cases (40%) of Stage III and 1 (3.33%) was Stage I.



Graph 1: Comparison of CD163 tumor-associated macrophages with the nodal status of oral squamous cell carcinoma

According to the Bryne's grading system, 10 (33.33%) cases of OSSC were categorized as Grade I (Well differentiated), 15 (50%) cases fell under the category of Grade II (Moderately differentiated) and 5 (16.67%) cases were Grade III (poorly differentiated).

On comparison of CD163 TAM score with TNM stages of OSCC, we observed that the CD163 TAM score increased from Stage I (49.0) to Stage II (69.21). However, a slight reduction was noted in its score in Stage III (64.6). The difference was not found to be statistically significant ($P > 0.05$).

Pairwise comparisons showed that there was no statistically significant difference between the CD163 TAMS among stages of OSCC ($P > 0.05$) [Table 1 and Graph 2].

On the correlation of TNM stages of OSCC with CD163 TAM score by Karl Pearson's correlation test, a positive correlation was found, that is, there was an increase in CD163 TAMs in higher TNM stages [Table 2].

On comparison of CD163 TAM score with histological grades of OSCC, we observed that the CD163 TAMs decreased from well-differentiated OSCC (73.8) to moderately differentiated OSCC (59.39) but increased in poorly differentiated OSCC (74.33). The difference in CD163 TAMs in different histological grades of OSCC was not statistically significant ($P > 0.05$)

On analysis of the difference between CD163 TAM score in the different histological grades of OSCC, we found a statistically significant difference only in CD163 TAMs of well-differentiated OSCC Vs. moderately differentiated OSCC.($P < 0.05$) [Table 3 and Graph 3].

The CD163 TAMs were found to decrease in the presence of nodal involvement of OSCC, N_0 (68.09) and N (64.6) cases. However, the values were not statistically significant ($P > 0.05$) (Graph 1).

Table 1: Comparison of CD163 Tumor associated macrophages with tumor, node, metastasis stages of oral squamous cell carcinoma by Kruskal-Wallis ANOVA and pairwise comparison by Mann-Whitney U-test

TNM stage	CD163 TAMs		
	Mean	SD	Sum of ranks
Stage I	49	0	6
Stage II	69.21	17.42	296
Stage III	64.6	14.26	163
Total	66.7	16.15	
H		2.5367	
P		0.2813	
Pair wise comparisons by Mann-Whitney U-test			
Stage I versus Stage II		*P*=0.9999	
Stage I versus Stage III		*P*=0.9999	
Stage II versus Stage III		*P*=0.2496	

*$P<0.05$. TAMs: Tumor associated macrophages, SD: Standard deviation, TNM: Tumor, Node, Metastasis

Table 2: Correlation between tumor, node, metastasis stages of oral squamous cell carcinoma with CD163 Tumor associated macrophages scores by Karl Pearson's correlation

Variables	Valid *n*	Correlation between TNM stages (Spearman R)
CD163-TAMS	30	0.0905

TAMs: Tumor associated macrophages, TNM: Tumor, Node, Metastasis

Table 3: Comparison of CD163- Tumor-associated macrophages with histological grades of oral squamous cell carcinoma by Kruskal-Wallis ANOVA and pairwise comparison by Mann-Whitney U-test

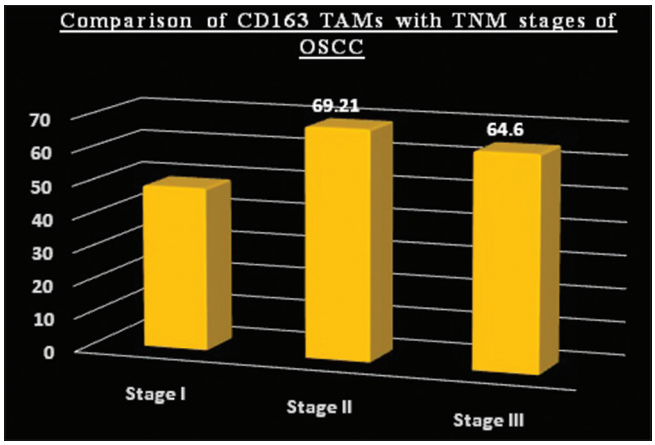
Histological grades	CD163 TAMs		
	Mean	SD	Sum of ranks
Well differentiated SCC	73.83	15.76	195.5
Moderately differentiated SCC	59.39	15.91	181
Poorly differentiated SCC	74.33	6.76	88.5
Total	66.7	16.15	
H		4.7133	
P		0.0948	
Pairwise comparisons by Mann-Whitney U-test			
WDSCC versus MDSCC		*P*=0.0520*	
WDSCC versus PDSCC		*P*=0.5006	
MDSCC versus PDSCC		*P*=0.1498	

*$P<0.05$. SCC: Squamous cell carcinoma, WDSCC: Well-differentiated squamous cell carcinoma. MDSCC: Moderately differentiated squamous cell carcinoma, SD: Standard deviation, TAMs: Tumor-associated macrophages, PDSCC: Poorly differentiated squamous cell carcinoma

DISCUSSION

The tumor mass is undoubtedly a multifaceted show with tumorigenesis as a complex multistep process, involving not only genetic and epigenetic changes in the tumor cell but also selective supportive conditions of the deregulated tumor microenvironment. It is a dynamic network that includes the cancer cells, stromal tissue, as well as the extracellular matrix (ECM) that surrounds it all.[20,21] TAMs represent a significant component of tumor microenvironment and constitute up to 50% of tumor mass.[15] TAMs are considered to be a distinct M2 polarized population promoting tumor progression.[11,22] CD163 is regarded as a highly specific monocyte/macrophage marker for M2 macrophage.[18]

There has been substantial clinical and experimental evidence that TAMs orchestrate all the stages of tumor progression.[5,11,23,24] M2 TAMs produce many immunomodulatory molecules including checkpoint inhibitors, and factors promoting angiogenesis[25] and tissue reconstruction, thus exerting trophic effects that exerts stromal angiogenesis, matrix breakdown[7,21] due to cleavage of E-cadherin-β-catenin complex and consecutive activation of Wnt-β-catenin pathway which is the main inducer of EMT[11,26,27] leading to tumor progression.



Graph 2: Comparison of CD163 tumor-associated macrophages with tumor, node, metastasis stages of oral squamous cell carcinoma

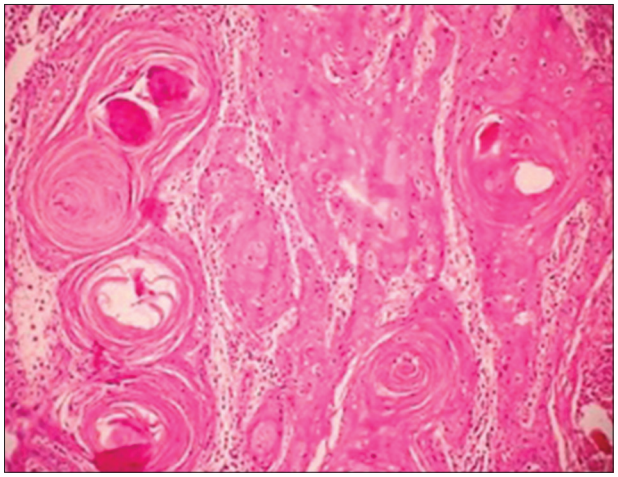


Figure 1: Well-differentiated squamous cell carcinoma H&E (×10)

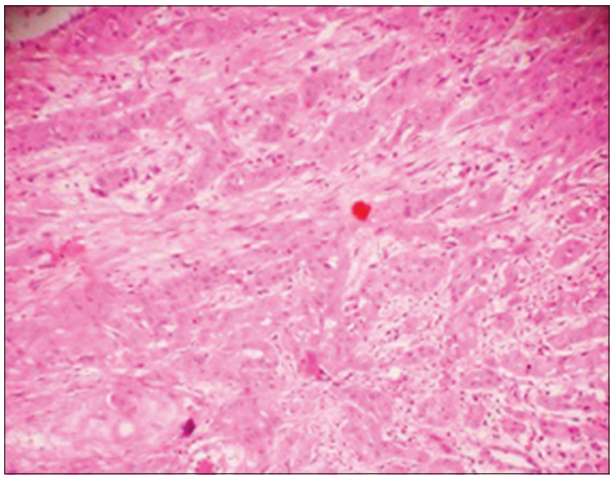
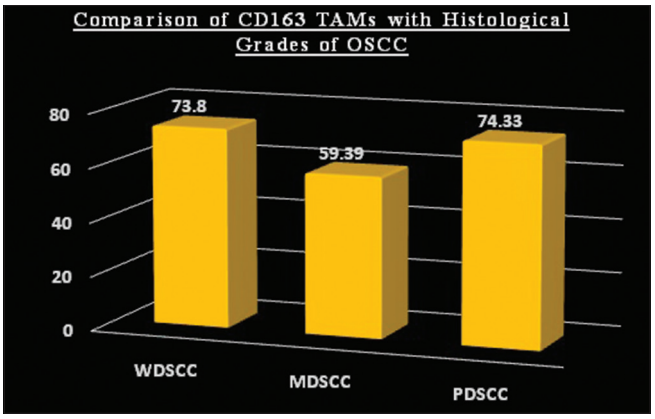


Figure 3: Moderately differentiated squamous cell carcinoma H&E (×10)

Thus the aim of this study was to evaluate the density of TAMs using CD163 in histological grades of OSCC.

On comparison of CD163 TAMs score in clinical stages of OSCC, the expression increased from Stage I (49.0) to Stage II (69.21). However, a slight reduction was noted in its score in Stage III (64.6).



Graph 3: Comparison of CD163 tumor-associated macrophages with histological grades of oral squamous cell carcinoma

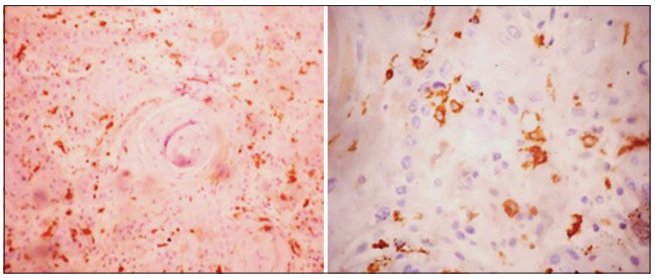


Figure 2: Immunohistochemical staining with Cd163 × 10, ×40 [Well Differentiated Carcinoma]

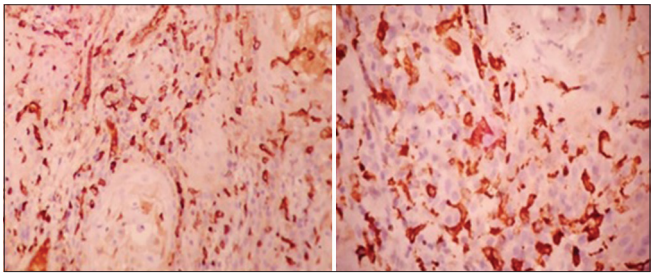


Figure 4: Immunohistochemical staining with Cd163 × 10, ×40 [Moderately Differentiated Carcinoma]

The results were not statistically significant ($P > 0.05$) [Table 1 and Graph 2]. On the correlation of TNM stages with CD163 TAM score, a positive correlation was found, that is, there has an increase in CD163 TAMs in higher TNM stages [Table 2].

Our results were in concordance with the study conducted by Fujii *et al*.[20] and He *et al*.[28]

This may be attributed to macrophage balance hypothesis, which states that in the early stages of carcinogenesis, innate responses are beneficial to the host and involve activation of effective surveillance

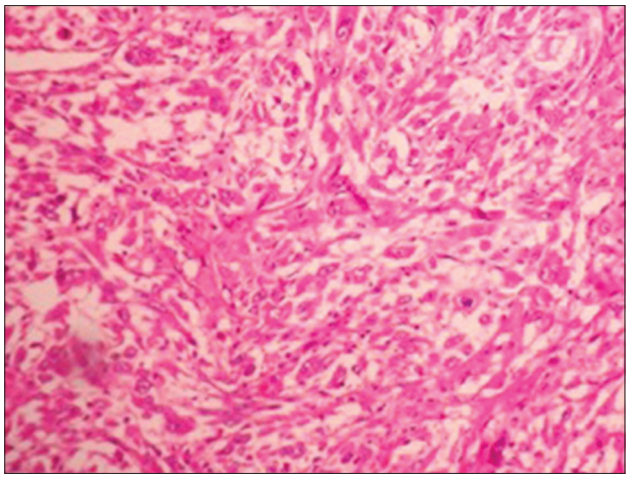


Figure 5: Poorly differentiated squamous cell carcinoma H&E (×10)

by adaptive immunity to eliminate tumor cells; while in established malignancy, TAMs orchestrate "smoldering inflammation" that promotes tumor progression.[12]

On comparison of CD163 TAM score expression with histological grades of OSCC, we found the highest expression in poorly differentiated OSCC (74.33). However, CD163 TAMs decreased from well-differentiated OSCC (73.8) to moderately differentiated OSCC (59.39). The difference was not found to be statistically significant ($P > 0.05$) [Table 3 and Graph 3]. On intragroup comparison, there was a statistically significant difference in CD163 expression between well-differentiated OSCC and moderately differentiated OSCC ($P < 0.05$).

Our results are in accordance with that of Fujii *et al*.[20] However, Mori *et al*.[29] found the CD163 expression increased with histological grades of OSCC with statistically significant values.

The high CD163 TAMs expression in poorly differentiated OSCC can be explained by the hypothesis that higher grades of tumor may facilitate differentiation of TAMs into the M2 phenotype by producing cytokines such as IL-10, IL-13, VEGF and chemokines such as CCL2.[22,23] However, the macrophage balance hypothesis may explain the low expression in MDSCC.

The CD163 TAMs score was found to decrease in the presence of nodal involvement of OSCC. There was an inverse relationship of CD163 TAMs with regional nodal involvement. However, the results were not statistically significant ($P > 0.05$) [Table 4].

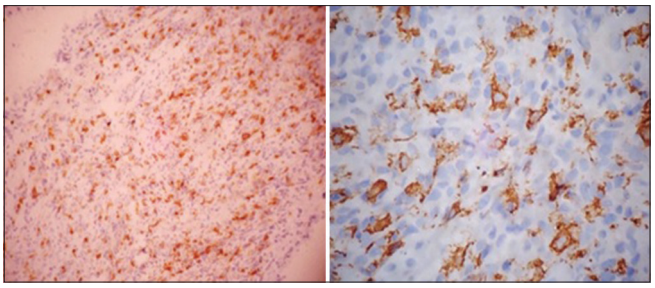


Figure 6: Immunohistochemical staining with Cd163 ×10, ×40 [Poorly Differentiated Carcinoma]

Table 4: Pairwise comparison of CD163 Tumor-associated macrophages with the nodal status of oral squamous cell carcinoma by Mann-Whitney U-test

N stage	CD163 TAMs		
	Mean	SD	Sum of ranks
No	68.09	17.55	302
n	64.6	14.26	163
Total	66.7	16.15	
Z		−0.9737	
P		0.3302	

$P<0.05$. TAMs: Tumor-associated macrophages, SD: Standard deviation

Our results are in accordance with that of Fujii *et al*.,[20] who observed high CD163 TAMs in early nodal involvement than late nodal involvement.

However, Mori *et al*.[29] found that CD163 TAMs increased with nodal involvement and was statistically significant.

Our study is novel in the sense that it highlights the effect of a component of the tumor microenvironment, TAMs, on the tumor cell.

In the present study, CD163 TAMs were evaluated in the normal oral mucosa. The values of CD163 TAMs ranged from 5 to 12, which were significantly less than that found in the cases of OSCC (44–98 average). Thus in our study, the CD163 TAMs values were significantly higher in OSCC than in normal mucosa. This was in concordance with Fujii *et al*. study.[20]

Although the study establishes a hopeful platform for the correlation between tumor cells and component of tumor microenvironment Cd163-positive TAMs, there is still a vast amount of information that remains to be discovered for the correlation between the two.

More extensive studies to link the two entities need to be carried out on cytokines produced by cancer cells that convert pro-inflammatory macrophages to anti-inflammatory macrophages.

CONCLUSION

Most of the OSCC patients in the present study were

in the age group of 40–60 years, with a mean age of 48.5 years. There was a definite male predilection noted with the majority of the patients being males - 27 (90%) out of a total of 30 cases. Gingivobuccal sulcus was the most common site for the occurrence of OSCC in this study.

As TAMs increased in higher clinical stages and histological grades of OSCC, it may be considered as an independent factor for determining the progression of the tumor.

Thus, the association between CD163 and grades of OSCC can be used to better define the prognosis of each patient, which will more accurately reflect the biological behavior and progression of each tumor.

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Conflicts of interest
There are no conflicts of interest.

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