THE ROLE OF TUMOR-ASSOCIATED MACROPHAGES IN THE PATHOGENESIS OF ORAL SQUAMOUS CELL CARCINOMA CORRELATED WITH THE CLINICOPATHOLOGICAL PARAMETERS

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ABSTRACT

INTRODUCTION: Oral cancer is a major health problem, causing high morbidity and mortality rates. Oral squamous cell carcinoma (OSCC) accounts for 90-95% of all oral malignancies. During the last decade, significant evidence has suggested that inflammatory tumor microenvironment (TME) plays an important role in tumorigenesis. The inflammatory cells and their signals are indispensable participants in the neoplastic process, fostering proliferation, survival, and migration of cancer cells. The macrophages are the most abundant and important stromal cells in the TME, which orchestrate the inflammatory response. They control the cellular proliferation and survival by stimulating the immune cells and by promoting integrated processes of inflammation and tissue repair. Therefore, the tumor associated macrophages (TAMs) may be of great prognostic significance in different types of tumors.

OBJECTIVES: This study was conducted to assess the TAMs density in OSCC using CD163 in metastasizing and non-metastasizing OSCC. Moreover, the correlation of TAM density with the lymph node status and the different histopathological grades of OSCC was assessed.

MATERIALS AND METHODS: The density of TAM was calculated in 30 surgical specimens taken from OSCC patients. Biopsies were taken from the primary tumor of 15 cases with lymph node metastasis and 15 cases with no lymph node metastasis. Fifteen normal mucosal tissues were taken from healthy individuals indicated for alveoloplasty as a control group. Immunohistochemical staining using the CD163 antibody was performed, using Labeled Strept-Avidin Biotin complex method. Positive cells were counted using the Image J free software package.

RESULTS: CD163 was expressed in human OSCC and the TAMs count was significantly correlated with lymph node metastasis and with the tumor differentiation. Higher density was detected in metastatic tumors and in the poorly differentiated OSCC than in the well and moderately differentiated cases.

CONCLUSIONS: CD163 positive TAMs could be a prognostic factor in OSCC cases as TAM density was significantly correlated with the lymph node status and the grade of differentiation of OSCC.

KEYWORDS: Oral squamous cell carcinoma, TAMs, CD163, Metastasis, Inflammation.

INTRODUCTION

Cancer of the oral cavity and oropharynx is a world health problem, with a nearly annual occurrence of almost 443,000 new cases and 241,450 deaths globally (1). There is an obvious increase in cancer incidence in the Middle East and North African (MENA) countries. Recently, it is reported to be one of the most important cause of death following cardiovascular diseases, infectious diseases, and traumatic injuries. It has been estimated that approximately 270,000 people die every year from cancer in this region (2).

Oral squamous cell carcinoma (OSCC) is taken into account the most common oral growth and representing for over ninetieth of all oral malignancies (3). The tumor microenvironment (TME) is essential for cancer initiation and metastasis (4). It comprises innate immune cells (macrophages, neutrophils, mast cells, and dendritic cells), adaptive immune cells (T and B lymphocytes), in addition to the cancer cells and their encompassing stroma. Macrophages are the most prominent immune cells within the TME which is considered to have a crucial role in tumor progression (5,6).

Macrophages are categorized into M1 and M2 subtypes. M1 macrophages (pro-inflammatory) are activated by interferon gamma (IFN-γ) and microorganisms. They express high levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin IL-6, IL-12, and major histocompatibility complex class II (MHC class II). Therefore, they are capable of killing pathogens and promoting antitumor immune reaction. In contrast, M2 macrophages (anti-inflammatory) are stimulated by IL-4, IL-10, and IL-13. They show reduced MHC class II and IL-12 expression. Most M2 macrophages therefore, are considered as tumor associated macrophages (TAMs) (7).

The growth factors and cytokines produced by TAMs facilitate the cancer cell proliferation and metastasis through induction of angiogenesis and lymphangiogenesis. The role of TAMs in lymphatic metastases is concentrated...
CD163 could be a specific biomarker of the M2 phenotype that can be used to detect M2 from M1 macrophages (11). Though, there are several studies concerning the positive correlation between the TAM density and the poor prognosis in numerous carcinomas (12,13), very little is understood regarding its significance in OSCC. The aim of the present work was to investigate and correlate TAMs density with the lymph node status and grade of differentiation of OSCC using CD163 antibody.

**MATERIALS AND METHODS**

**Histopathological Examination**

Biopsies taken from the tumor tissues were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax using the conventional procedures. Serial sections of 3-4 μm thickness were placed on glass slides and stained using Hematoxylin and Eosin (H&E) for histological evaluation and grading of OSCC.

**Immunohistochemical analysis**

Specimens of tumor tissue from the study groups were stained using rabbit monoclonal antibody CD163 (EP324; cat #RM0027: 1.0 ml). Immunostaining was performed on 4 μm paraffin sections. Sections were de-paraffinized with xylene and rehydrated in graded ethyl alcohol. Before the staining procedure, samples were immersed in citrate buffer solution (pH = 6). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. Then, sections were cooled for 20 minutes. Sections were incubated with primary antibody (diluted 1:100; catalog no. RM0027) for 1 hour at room temperature. Finally, secondary antibody associated with Ultra Vision detection system was applied for 30 minutes at room temperature. Sections were washed in PBS again. The 3,3′-diaminobenzidine was applied as a chromogen for staining procedure, samples were immersed in citrate sections were incubated with primary antibody (diluted 1:100; catalog no. RM0027) for 1 hour at room temperature and then were washed in phosphate-buffered saline (PBS). Finally, secondary antibody associated with Ultra Vision detection System was applied for 30 minutes at room temperature. Sections were washed in PBS again. The 3,3′-diaminobenzidine was applied as a chromogen for antibody detection. Sections were counterstained with Mayer’s hematoxylin and covered with glass slip.

**Histopathological Results**

Thirty cases were diagnosed as OSCC histologically using H & E stain. The microscopic examination revealed 9 cases (30%) of the well differentiated type, 14 cases (46.67%) were moderately differentiated OSCC, and 7 cases (23.33%) were poorly differentiated type. Figure 1 reveals detailed grades of OSCC and their histopathological features.

**Statistical Analysis**

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp). Qualitative data were described using number and percent (16). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean (±standard deviation), and median. In all the statistical results, a p value < 0.05 was considered significant. The F-test (ANOVA) was used for normally distributed quantitative variables to compare between more than two groups, while the Post Hoc test (Tukey) was used for pairwise comparisons.

**RESULTS**

**Clinical Results**

The demographic data of the patients included in the current study is shown in Table 1. The current study showed that the age of the patients ranged from (35-76 years). The mean age was found to be (57 years). Thirteen patients (43.33 %) were females and 17 (56.66 %) patients were males. The most common site of occurrence was the lateral side of the tongue, representing 40 % of the cases (n=12), while buccal mucosa was the second common site, representing 20% of the cases (n=6), followed by the floor of the mouth (16.66 % n=5), then the alveolar mucosa (n=3) and the palate (n=3) represented 10% each, and only one case (3.33%) was found on the lower lip.

**Distribution of the Studied OSCC Cases According to the Demographic Data.**

| Age | (%) |
|-----|-----|
| ≤60 | 16  |
| >60 | 14  |

**Gender**

| Gender | (%) |
|--------|-----|
| Male   | 17  |
| Female | 13  |

**Clinical Variants**

| Clinical Variants | (%) |
|-------------------|-----|
| Ulcerative        | 18  |
| Leukoplakic       | 5   |
| Exophytic         | 7   |

**Correlation of TAMs density with lymph node status**

on their ability to provide pro-lymphangiogenic factors like vascular endothelial growth (VEGF)-C and (VEGF)-D that stimulate the growth of intratumoral lymphatic vessels (8,9). They additionally release matrix-remodeling enzymes including MMP-2, MMP-9, and urokinase plasminogen activator (UPA) that are known to contribute to lymphangiogenesis (8). Additionally, they promote an immunosuppressive environment with other immune cells that helps tumor to grow unlimitedly (10). and the immunohistochemically positive cells were counted using the Image J free software package.
Lymph node metastasis

|          |       |
|----------|-------|
| Positive | 15    |
| Negative | 15    |

Figure (1): (A-C) A-Well Differentiated OSCC Shows hyperchromatic and pleomorphic malignant epithelial cells forming large keratin and epithelial pearl (H&E stain x 400)  
B-Moderately Differentiated OSCC Revealing Hyperchromatic Large Bizzar Nucleus (arrow), Cellular and Nuclear Pleomorphism, Hyperchromatism and Loss of Desmosomes in the Malignant Epithelial Cells Forming the Cell Nests (H&E stain x400).  
C-Poorly differentiated OSCC showing nuclear hyperchromatism, cellular and nuclear pleomorphism (arrow), altered nuclear cytoplasmic ratio in the highly anaplastic epithelial cells (H&E stain x 400)

Immunohistochemical Results
All the studied cases (100%) showed positive immunoreactivity to CD163, which strongly stained the M2 macrophages. Whereas, the control cases of the normal oral mucosa showed negative immunoreactivity for CD163 (Figure 2).

Figure (2): Normal oral mucosa showing negative immunoreactivity for CD163 (immune stain X 100)

Macrophages were observed primarily in the tumor stroma and between the tumor cells. However, very few numbers of TAMs resided in the tumor nests. The positive CD163 immune-staining showed a cytoplasmic expression with an accentuation of the plasma membrane, and no nuclear reaction. The shape of CD163 positively stained TAMs was predominantly spindle, with few rounded and angular cells (Figure 3).

Figure (3): (A, B) A- Moderately Differentiated OSCC Showing Numerous Number of TAMs Peritumoral with a Few Number Intratumoral (immuno stain of CD163 x 400)  
B- Moderately differentiated OSCC showing a cytoplasmic expression pattern of CD163. Notice the spindle shape of the stained cells. (immune stain of CD163 x400)

Concerning TAMs density, higher density was detected in the cases with positive lymph nodes than the cases with negative lymph nodes (Figure 4).

Figure (4):
Correlation of TAMs density with lymph node status

The 30 cases (100%) presented a certain degree of immunoreactivity for CD163. The average number of TAMs per mm$^2$ (TAMs density) for the lymph node positive OSCC cases was $26.19 \pm 6.97$, while it was $9.83 \pm 3.73$ for the lymph node negative cases. The difference in the mean TAM density between the lymph node positive and negative groups using (student t-test) was statistically significant ($P < 0.001$, Table 2).

Table (2): Comparison between the two studied groups according to TAM density.

| TAM Density          | Lymph node status | t   | p        |
|---------------------|-------------------|-----|----------|
|                     | Positive (n=15)   |     |         |
| Min. – Max.         | 18.0 – 41.0       | 8.014 | <0.001* |
| Mean ± SD.          | 26.19 ± 6.97      |     |         |
| Median              | 24.0              |     |         |
|                     | Negative (n=15)   |     |         |
| Min. – Max.         | 4.30 – 15.70      |     |         |
| Mean ± SD.          | 9.83 ± 3.73       |     |         |
| Median              | 9.60              |     |         |

Figure (4): A- Moderately Differentiated OSCC with Positive Lymph Node Revealing Increased Number of Stained TAMs (Immuno Stain of CD163 x400).
B- Moderately Differentiated OSCC with Negative Lymph Node Showing decreased number of TAM encircling the malignant epithelial nests (Immuno Stain of CD163 x400).

Regarding the histological grading, the number of TAMs in the well differentiated OSCC cases was relatively lesser than the number in moderately differentiated cases. Furthermore, the same finding was found comparing the moderately differentiated cases with the poorly differentiated ones, where the poorly differentiated cases had greater number of TAMs than the moderately differentiated cases (Figure 5).

Correlation of Tumor associated macrophages density with histological grading

The average number of TAMs per mm2 for all the poorly differentiated cases was $24.54 \pm 12.62$, while in the well differentiated and moderately differentiated cases, the average number of TAMs was $14.19 \pm 8.72$ and $19.84 \pm 7.86$ respectively. The difference between the mean TAM density between the well, moderately, and poorly differentiated groups using t-test (ANOVA) was statistically significant ($P < 0.05$) (Table 3).

Table (3): Relation between histological grade and TAM count in studied groups.

| TAM density | Histological grade | F     | p       |
|-------------|--------------------|-------|---------|
|             | Poor (n=4)         |       |         |
| Min. – Max. | 13.40 – 15.70      | 38.649| <0.001* |
| Mean ± SD.  | 14.80 – 15.0        |     |         |
| Median      | 14.60              |     |         |
| Sig. bet. grades | p1<0.001, p2<0.001, p3=0.004 |       |

Table (3): Relation between histological grade and TAM count in studied groups.

| TAM density | Histological grade | F     | p       |
|-------------|--------------------|-------|---------|
|             | Moderate (n=6)     |       |         |
| Min. – Max. | 7.30 – 12.0        | 51.849| <0.001* |
| Mean ± SD.  | 9.36 – 9.40        |     |         |
| Median      | 8.95               |     |         |
| Sig. bet. grades | p1<0.001, p2<0.001, p3=0.004 |       |

Table (3): Relation between histological grade and TAM count in studied groups.

| TAM density | Histological grade | F     | p       |
|-------------|--------------------|-------|---------|
|             | Well (n=4)         |       |         |
| Min. – Max. | 4.30 – 5.40        | 51.849| <0.001* |
| Mean ± SD.  | 4.77 – 4.60        |     |         |
| Median      | 4.60               |     |         |
| Sig. bet. grades | p1<0.001, p2<0.001, p3=0.004 |       |

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the three categories
p1: p value for comparing between Poor and Moderate
p2: p value for comparing between Poor and Well
p3: p value for comparing between Moderate and Well
*: Statistically significant at p ≤ 0.05

DISCUSSION

In Egypt, the relative incidence of oral cancer in 2014 was 0.9% in males and 0.75% in females (17). The prognosis
of OSCC remains disgraceful as more than 50% of the patients die within 5 years due to the late diagnosis (18,19). Therefore, an improved comprehension of the cellular and molecular mechanisms, which initiate tumorigenesis or promote cancer progression has taken the attention as a novel cancer treatment approach. Recently, there has been a growing recognition of interest in the anti-tumor functions initiated by the innate immune response. Macrophages in cancers have an anti- or pro-tumoral effect on the tumor microenvironment (20). The present study evaluated the possible relationship between TAMs density in the primary tumor of OSCC with the lymph node status and histological grading of the lesion.

In the current study, macrophage infiltration into the OSCC was evaluated by using CD163 antibody which was expressed in all the studied cases of OSCC. No expression was found in the normal mucosal tissues and hyperplastic lesions. These results were in consistence with those conducted by Yamagata et al., (14), Wang et al., (21) and Mori et al., (22) who confirmed the dominance of M2 macrophages in OSCC by the positive immune-expression of CD163, while the negative immune stain of CD163 in the normal tissues has suggested that M2 macrophages are not the predominant components in these tissues.

Carcinomas are known to metastasize to the regional lymph nodes, with crucial role of TAMs in producing pro-lymphangiogenic factors enhancing the metastases. In the present study, a high TAMs density was significantly correlated with positive lymph node metastasis. This result was in agreement with Yamagata et al., (14) and Wang et al., (21), who reported that metastasizing OSCCs had a significantly higher TAMs density than non-metastasizing lesions. Similarly, they observed the presence of TAMs within the tumor mass and in the peritumoral areas.

The present work revealed that CD163+ TAMs count was significantly correlated with the tumor grade. The highest and lowest counts of TAMs were associated with poorly and well differentiated histological grades, respectively. Therefore, TAMs density could be correlated with the progressive cell transformation in OSCC. This was in accordance with many studies (17, 23-25), which confirmed that the infiltration of TAMs type M2 in OSCC was correlated with tumor aggressiveness and poor prognosis.

Although the exact reason for the increased M2 macrophages in higher-grades is currently unknown, several potential mechanisms were proposed. First, the tumor microenvironment of the high grade tumors may facilitate differentiation of macrophages into the M2 phenotype by producing cytokines and chemokines such as IL-10 and IL-13. Furthermore, CCL2 known as monocyte chemoattractant protein-1 and IL-6 promote the survival of human monocytes and induce M2- macrophage differentiation (25). They were found to be highly expressed in many cancer types, including OSCC, whereas normal oral mucosal epithelial cells expressed lower levels of these cytokines and chemokines (26,27). Therefore, the tumor microenvironment especially of the higher grades may exhibit increased levels of various cytokines and chemokines that might promote monocyte/macrophage survival and differentiation into the M2 phenotype.

Contradictory results were found by Forssell et al., (28) on colorectal cancer. They found that the density of TAMs, especially the peritumoral cells, was inversely correlated with the tumor grade, clinical stage and patients’ survival. This was explained by the dominance of tumoricidal macrophages (M1 type) at the peritumoral sites, where TAMs are less exposed to hypoxia and tumor-derived cytokines. Therefore, they may differentiate into M1 type and secrete cytotoxic molecules such as Reactive Oxygen Species (ROS), Nitrogen Oxides (NO) and Tumor Necrosis factor α (TNF-α), which are able to kill the tumor cells and induce cancer cell apoptosis. Furthermore, Ming et al., (29) found that no significant association was detected between TAMs and the tumor grade. The discrepancy between the present data and the previous reports may be due to the limited sample size and the presence of few cases of the poorly differentiated grade.

The results of the current study supported the possibility of using TAMs density in the tumor to point out the OSCC patients who are at risk of developing cervical lymph node metastasis. This parameter could be useful for selecting head and neck squamous cell carcinoma patients who are more susceptible to show lymphatic metastasis to undergo elective cervical lymph node dissection. Furthermore, new therapeutic approaches against tumors based on inhibition of TAMs recruitment or suppression of TAM survival are under investigation. Other possibilities in fighting of TAMs would be blocking of the pathways associated with the protumor M2 phenotype or reprogramming of TAMs into the antitumor M1 phenotype. Therefore, the increasing knowledge about the biological effects of TAMs and the tumor microenvironment in OSCC may lead to novel cancer therapies (30–32).

CONCLUSION

Tumor associated macrophages density was significantly correlated with lymph node status and the histological grading of OSCC. So, it could be used as an indicator for progression and early metastasis of OSCC.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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