Dentistry Section

Quantitative Assessment of Tumor Associated Macrophages in Head and Neck Squamous Cell Carcinoma Using CD68 Marker: An Immunohistochemical Study

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ABSTRACT

Introduction: Oral Squamous Cell Carcinoma (OSCC) is one of the most prevalent cancers in India. Clear evidence regarding inflammation being an etiological factor of cancer was found only in the last few decades. A major inflammatory component in the tumor tissue is Tumor-Associated Macrophages (TAMs). The CD68 antibody is a marker for staining TAMs.

Aim: The aim of this study is to quantify the macrophage count in healthy oral mucosa and OSCC and comparing TAMs in different histopathological grades of OSCC immunohistochemically.

Materials and Methods: Thirty archival specimens of OSCC patients and 10 healthy biopsy samples were collected. Immunohistochemical staining was done using a CD68 marker.

Statistical analysis was done using Kruskal-Wallis ANOVA and Mann-Whitney U test.

Results: Comparing CD68 expression in various study groups showed a significant difference (p=0.000). The pair-wise analysis showed different grades of OSCC, which differed significantly for CD68 expression from the normal oral mucosa.

Conclusion: The most significant cells present in tumor stroma are TAMs, which remain in close proximity to neoplastic cells and interact with them via several chemical mediators, which may serve to increase the invasiveness of the malignant epithelium. Dense infiltration of TAMs adjacent to tumor cells and islands vividly implies their role in tumor progression.

INTRODUCTION

Oral Cancer (OC) occurring in India accounts for 57.5% of all global occurrences [1]. The European Union registers about 40,000 new cases per year while 30,000 new cases are registered annually in the United States [2]. In South-Asia OSCC is found to be the most common cause of cancer-related deaths [3]. This high prevalence is mainly because of region-specific epidemiological factors, like tobacco and betel quid chewing,

The first possible link between cancer and an inflammatory tissue microenvironment was noticed by Rudolf Virchow in the 19th century, but clear evidence regarding the role of inflammation was found only in the last few decades [4]. It has been observed that along with promoting tumor development, tobacco, also produces chronic inflammation which facilitates tumorigenesis [5].

One of the major inflammatory components in the tumor tissue is TAMs. Macrophages can be grouped into two types, one that is normally present in inflamed tissue (M1 phenotype) and the other that is present in cancer-related inflammation (M2 phenotype). The classical M1 phenotype macrophages are part of the immune system, intricately involved in processes such as phagocytosis and production of inducible Nitric Oxide Synthase (iNOS) and Reactive Oxygen Species (ROS) serving to protect the organism from harmful pathogens. On the other hand, macrophages that are of the M2 prototype are produced by chemokines and polarizing cytokines, released by tumor cells and thus are able to evade the immune system ensuring their escape from destruction and subsequently they proliferate [6]. Thus, the aim of the study was to evaluate and quantify CD68 antibody (a marker for staining TAMs) in normal tissue and OSCC using immunohistochemistry.

MATERIALS AND METHODS

Thirty archival (excisional biopsy) specimens of formalin-fixed-paraffin-embedded tissue blocks of OSCC patients were retrieved from the Department of Oral Pathology & Microbiology, Dr. D. Y. Patil Dental College & Hospital, Pune, for the study. Sections were stained by H & E to differentiate between different grades of OSCC [Table/Fig-1-3]. Ten biopsy samples for the control group were obtained from patients undergoing esthetic gingivoplasty, (after thorough oral prophylaxis and reduction of gingival inflammation). The study was approved by the Scientific and Ethical Committee of the Institution. Written informed consent was obtained from the patients prior to taking his/her tissue for this study.

Immunohistochemical Staining: A 5μm-thick paraffin section was taken on lysine-coated slides and was stained immunohistochemically using mouse monoclonal antibodies to CD68 (Thermo Fisher Scientific, MS-397; Lab Vision Corporation, Fremont, CA, USA). Primary antibody was used in 1:200 dilutions (as per product instructions for use). Before treatment with the primary antibody, tissue sections were subjected to enzyme digestion for 5 minutes at 37°C with Protease XXV at 1mg/ml PBS [Lab Vision Catalog # AP 9004]. The CD68 stained slides so obtained were observed under a light microscope at low magnification (10X) [Table/Fig-4] for three 'Hot Spots' i.e. areas where the density of CD68 positive cells was recorded maximum by two observers NB and SR independently. The examiners were considered calibrated once a statistically significant correlation and statistically non-significant difference between duplicate measurements were obtained (r=0.92). These 'Hot Spots' were then seen under high magnification (40X) [Table/Fig-5] using a light
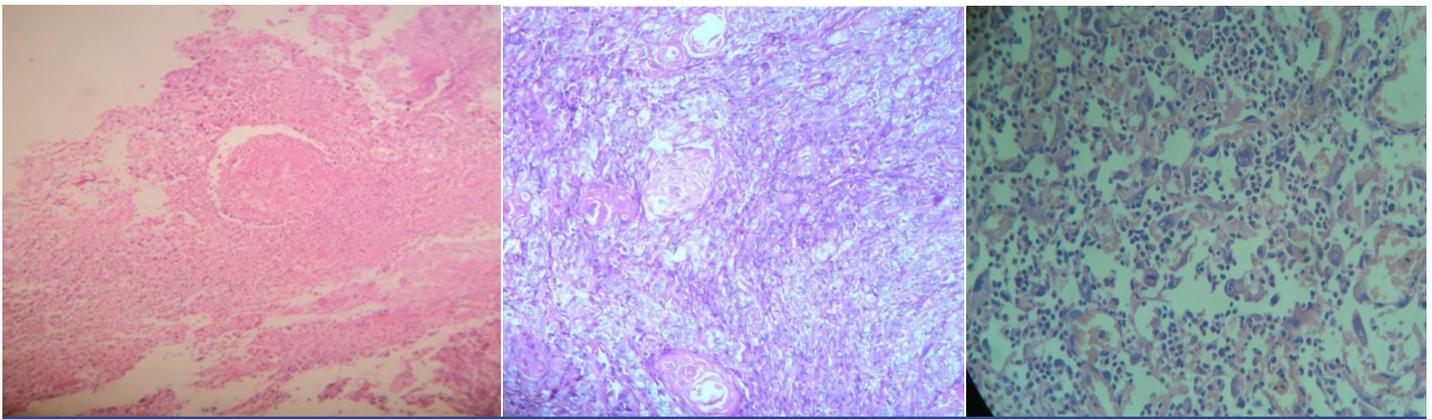
[Table/Fig-1]: H & E section of well differentiated squamous cell carcinoma with keratin pearl (10x). [Table/Fig-2]: H & E section of moderately differentiated squamous cell carcinoma (10x). [Table/Fig-3]: H& E stained section of a poorly differentiated oral squamous cell carcinomatous tissue (40x).
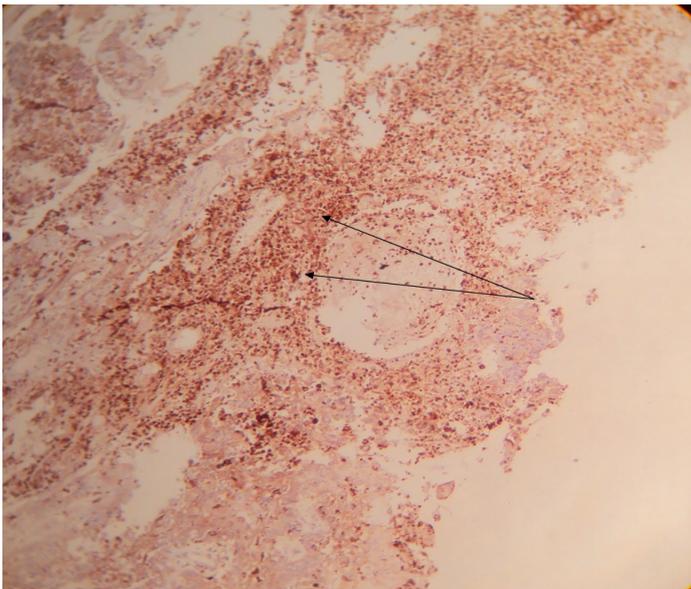
[Table/Fig-4]: A 10x magnification of a CD68 stained section of a well differentiated OSCC showing hot-spots (dense infiltration) of CD68 +ve tumor-associated macrophages (shown with arrows).
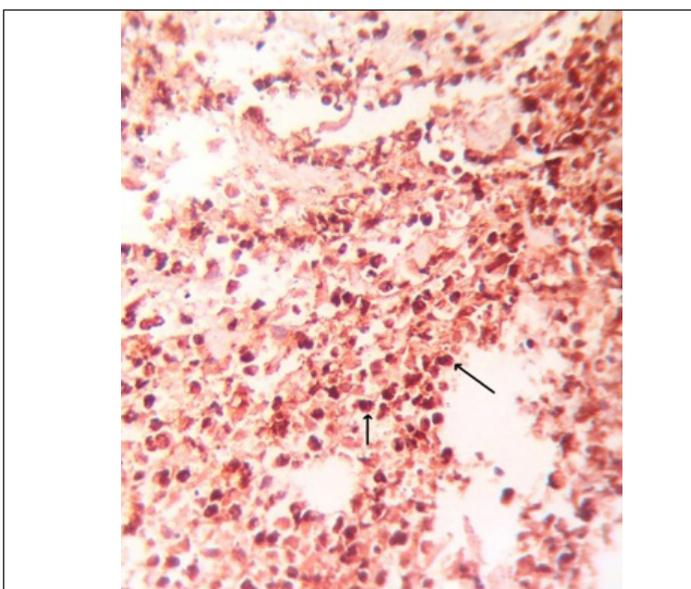
[Table/Fig-5]: A 40x magnification of a well differentiated OSCC showing CD68 positive tumor-associated macrophages (shown with arrows).

microscope and CD68 positive cells were counted and the mean value was obtained. Values thus obtained were graded as follows: Of the hundred cells counted, GRADE 0: Single few cells positive; GRADE+1: Less than 10% of the total number of cells in that hot spot were positive; GRADE+2: More than 10% & less than 50% cells positive; GRADE+3: More than 50% cells positive.

STATISTICAL ANALYSIS

All values were subjected to statistical analysis using Kruskal-Wallis ANOVA and Mann-Whitney U test. All statistical analyses were completed using SPSS version 20.0 software. A p value of <0.05 indicated statistical significance.

RESULTS

The comparison of CD68 expressions in the various differentiations of OSCC groups is tabulated using Kruskal-Wallis ANOVA [Table/Fig-6]. The Chi-Square value of 28.824 and p-value of 0.000 was observed which is statistically highly significant. The pair-wise analysis using Mann-Whitney U test [Table/Fig-7] showed that all the grades of OSCC differed significantly with respect to the expression of CD68 from the normal oral mucosa (control group), whereas, there was no statistical difference in the expression of the biomarker between the various grades of OSCC.

Group	N	Mean rank	Chi-square value	p-value
Well differentiated OSCC	10	27.00		
Moderately diffentiated OSCC	10	24.00	28.824	0.000**
Poorly differetiated OSCC	10	25.50		
Normal mucosa	10	5.50		

[Table/Fig-6]: Comparison of CD68 expression in the study groups using Kruskal Wallis ANOVA.
** Highly significant

Group	Mean rank	Mann Whitney U	p-value
Well differentiated OSCC	Moderately differentiated OSCC	40.00	0.276
	Poorly differentiated OSCC	45.000	0.542
	Normal mucosa	0.000**	0.000**
Moderately differentiated OSCC	Poorly differentiated OSCC	45.000	0.615
	Normal mucosa	0.000**	0.000**
Poorly differentiated OSCC	Normal mucosa	0.000**	0.000**

[Table/Fig-7]: Pair wise comparison of the study groups using Mann Whitney U test.
** Highly significant

DISCUSSION

A wide range of cells are found in the microenvironment of OSCC tissue, consisting of both epithelial (malignant) and stromal cells. It has been observed that the dynamic interplay between these cells is responsible for tumor progression. TAMs constitute a dominant portion of the leukocyte population in tumor stroma [7].

Areas of hypoxia will occur within a solid tumor which has grown more than 2mm due to insufficiencies in the simple diffusion of oxygen as well as nutrients to metabolizing tissues. In such areas, several factors such as Monocyte Chemotactic Protein 1 (MCP-1)

/ CCL2 and Granulocyte-Macrophage-Colony-Stimulating Factor (GMCSF) are produced which in turn help to recruit monocytes continually within the tumor microenvironment. These accumulate in hypoxic areas after they differentiate into TAMs [7,8].

The presence of TAMs was observed 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 certainly denote the recruitment of TAMs within the tumor microenvironment and their potential role in modifying the neoplastic biological behavior of the tumor. This may also suggest that the TAMs are involved in tumor cytotoxicity as well as scavenging of tumor cell debris.

Lu CF et al., in their study with 92 OSCC tissue specimens found the infiltrating macrophage count to be significantly higher in OSCC tumors with a large tumor size, positive lymph node metastasis and more advanced clinical stages or recurrence [8, 9]. Our findings are in accordance with them as we also found significant macrophage count in OSCC specimens and the tissues of patients with lymph node metastasis. Dai T et al., in their sample of 42 cases of OSCC and 10 normal tissues found that there were masses of macrophage infiltration in OSCC [9,10], which is in agreement with their previous findings as well as the findings in our study. El-Rouby DH in a study observed that the CD68 positive TAMs were distributed within the connective tissue surrounding the cancer cells [10,11] and similar observations were found in our study.

However, our findings are different from the study by Lo Muzio L et al., [11,12] with respect to tumor differentiation. We found no statistically significant difference in the expression of CD68 biomarker between the various histopathological grades of OSCC on using Mann-Whitney U test while their results showed a trend for the association of inflammatory infiltrates with the degree of tumor differentiation: well and moderately differentiated tumors showed associations of dense inflammatory infiltrate while poorly differentiated cancers seem to be associated to a low inflammatory infiltrate.

Since it is usually observed that TAMs are located in the stroma surrounding cancer cells, this localization of TAMs promotes angiogenesis, as a number of molecules with possible impact on angiogenesis have been shown to be expressed by macrophages in low oxygen conditions, such as Vascular Endothelial Growth Factor (VEGF), Tumor Necrosis Factor (TNF)- and Basic Fibroblast Growth Factor (bFGF) [7].

Mantovani A et al., have proposed that induction of TAMs and their polarization into the M2 phenotype may be caused due to the exposure of macrophages to IL4 and IL10 in tumor microenvironments [12, 13]. It has been shown that the invasive properties of tumor cells get enhanced when tumor cells are co-cultured with macrophages in a manner that is dependent on TNF-α and matrix metalloproteinases as reported by Hagemann T et al., [13, 14]. The proliferation of tumor cells and infiltration by TAMs have positive correlations in various carcinomas as shown in studies measuring Ki67 indices in endometrial carcinomas [14, 15], the mitotic index in renal cell carcinomas [15, 16] and levels of MIB-1 in breast carcinomas [16, 17]. It seems that TAMs play dual roles in the process of metastasis by aiding the release of the primary tumor cells that will metastasize and also in establishing the secondary tumor at a distant site [17, 18]. It has been shown that infiltration of TAMs was regulated by hypoxia inducing factor semaphoring [19]. Also, TAMs are responsible for cancer progression, they initiate cancer progression via 'angiogenic switch' by releasing proangiogenic cytokine VEGF-A. Formation of new blood vessels within the tumor microenvironment provides nutrition and oxygen to fast growing tumor cells, at the same time they help in the escape of tumor cells through the newly formed blood vessels which initiate metastasis [19]. Recent studies have

indicated a clear association of roles played by TAMs and Cancer Stem Cells (CSC). Markers of TAMs like CD163 have been found to be overexpressed along with CD68 in OSCC tissue compared to normal tissue and this suggests that CD68 is an important diagnostic and prognostic marker for OSCC [20]. An association between TAM and CSC markers has been established. The aggressiveness in biological behavior of the tumor can be implied by the expression of these markers [20]. There have been studies indicating that micro-localization of TAMs can serve as an important prognostic indicator in OSCC [21]. It has been observed in murine studies that the systemic depletion of macrophages in Polyomavirus Middle T Antigen (PyMT) induced mammary cancer, results in a decrease in lung metastasis indicating the role of TAMs in the process [22]. A meta-analysis study by Zhang QW et al., has found that TAMs seem to have an association with poor survival in gastric, urogenital, and head and neck cancer patients [23].

Recent work on TAMs indicates that strictly classifying them into two phenotypes M1 and M2 as mentioned before is an over simplification since they are not static [24] and phenotypical TAMs show plasticity and can display a number of activation states ranging between M1 and M2 [6]. However, infiltration of M1 macrophages is accompanied by an increase in the infiltration of macrophages with M2 phenotype. Hence, according to stage-dependent manner/grading they can be correlated with better prognosis [25]. 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 immunohistochemistry techniques need to be identified which will serve the purpose of identification and quantification of the entire range of phenotypically different TAMs. Moreover, correlating the TAM count with clinical findings may reveal the exact role of TAMs in the prognosis of the disease.

LIMITATION

Further investigations are needed to elucidate the mechanism for regulation of extracellular matrix by TAMs and other stromal cells in human malignancies, and this may help for the design of novel therapeutic strategies in future.

CONCLUSION

TAMs form a significant portion of tumor stroma and are mainly presented adjacent to the tumor cells. It has become clear now that within the tumor microenvironment, not only the neoplastic epithelial cells but also the stromal cells have a major role to play in the biological behavior and progression of cancer. The significance of TAMs which remain in close proximity to the tumor cells or interacting with the tumor cells with the help of several chemical mediators cannot be overlooked. The dense infiltration of TAMs adjacent to the islands of cancer cells vividly implies its role in tumor progression. Thus, only by improving our understanding of cells within the tumor stroma and its cellular elements can we hope to plan better and effective therapeutic strategies for the treatment of OSCC.

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