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Original Article

Relationship between Mast Cell Density and Microvessel Density in Oral Squamous Cell Carcinoma and Normal Oral Mucosa: Immunohistochemical Analysis using CD117 and CD34 Antibodies

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ABSTRACT

Introduction: Mast cells are reported to be linked with the promotion of tumorigenesis in several malignancies. Present study was aimed at determining any potential relationship between mast cells and angiogenesis in different grades of oral squamous cell carcinoma (OSCC) and comparing them to normal oral mucosa (NOM).

Methodology: In this analytical comparative cross sectional study, 40 tissue sections were examined, 20 cases were diagnosed as OSCC and other 20 as NOM. To measure mast cell density (MCD) and microvessel density (MVD), immunohistochemical analysis comprised reacting formalin-fixed, paraffin-embedded blocks with monoclonal CD117 and CD34 antibodies respectively was performed . Statistical analysis included a t-test, ANOVA, and Pearson correlation, and a p-value of < 0.05 and a 95% confidence interval were considered significant.

Results: In OSCC, the mean (SD) MCD and MVD were 22.4 (10.8) and 22.3 (7.48), respectively. MCD and MVD were shown to have a strong negative correlation (r = -.702, p- value =.001). In the grade 3 OSCC, the MCD was lower than the mean MCD found in NOM. MVD was found to have a positive correlation with tumor progression (r =.895, p-value =.001). The mean (SD) MCD and MVD in OSCC and NOM were 22.4 (10.8) and 11.5 (2.58), respectively, and no significant relationship between MCD and MVD was identified.

Conclusion: In OSCC, a negative correlation between MCD and MVD is reported. When compared to NOM .MCD is found to be significantly higher in OSSC in the initial stages, but it subsequently declines in later stages, raising the possibility of protective effect by immune regulation.

Keywords: Mast Cells; Oral squamous cell carcinoma; microvessel density; angiogenesis

Introduction

Oral squamous cell carcinoma (OSCC) accounts for over 90% of all oral cancers[1]. It is the sixth most common cancer in the world, accounting for nearly 4% of new reported cases and 2% of all cancer deaths each year[2]. Studies have shown a high incidence of OSCC in South Asian countries, particularly in Pakistan, where it is reported as the second most common cancer[3]. It is a major public health problem, with one of the highest fatality rates among all cancers, according to the World Health Organization (WHO) [4].

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Despite substantial advances in research, it is still linked with significant mortality and morbidity rates, owing mostly to late diagnosis[5]. The current classification of head and neck cancers supports the Broders grading system and distinguishes well, moderately, and poorly differentiated grades of OSCC. With a high degree of keratinization and well-formed keratin pearls, well-differentiated OSCC mimics normal squamous epithelium to a great extent[6].Tumor progression is a complex process and depends upon multiple factors. Development of new blood vessels is one of the key components of "angiogenesis," and it is critical for the local invasion and distant metastasis of OSCC[7] The foundation of this new vessel formation depends upon the balance between positive and

negative angiogenic modulators within the microvasculature[8].

Mast cells are histologically seen as ovoid to spindle-shaped cells with cytoplasmic granules and routinely exist in the connective tissue of all organs, notably surrounding blood vessels and nerves in the dermal layer of skin. They have a debatable part in immunohistopathology and tumor angiogenesis. They are considered multifunctional cells with local and systemic effects and do so by assisting the release of multiple effective mediators like histamine, leukotrienes, and cytokines[9]. They also occur in several pathological states where they play a remarkable role in moderating the release of angiogenic factors which promote the local growth of tumors and distant metastasis[10]. Aside from angiogenesis, they also cause immunosuppression, extracellular matrix disruption, and eventually accelerate tumor cell mitosis[11]. They also have mitogenic potential and can promote tumor development by deleting tumor suppressor genes and expressing certain oncogenes via the c-kit locus. 10 Mast cell anti-tumor effects are assumed to be mostly owing to their involvement in innate immunity; consequently, they play a contentious role as both pro and anti-tumorigenic[12].

Angiogenesis is linked to local neoplastic development and distant metastasis. Similarly, mast cells are thought to be independent prognostic indicators in a variety of malignancies. Regulating these factors in OSCC may aid in predicting prognosis and disease aggressiveness[13, 14].

The present study was aimed at determining the mast cell density (MCD) and microvessel density (MVD), as well as their potential correlation, in different OSCC tumor grades and compare them to the MCD and MVD of normal oral mucosa (NOM).

Materials and Methods

This analytical, comparative study was conducted at the department of histopathology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from January to December 2019. Ethical approval was granted by Institutional Review Board of Armed Forces Institute of Pathology (Ref No. IRB/513). Non-probability convenience sampling was utilized. A total of 40 formalin-fixed, paraffin-embedded blocks 20 oral squamous cell carcinoma (OSCC) and 20 normal oral mucosa (NOM) were included in the study sample. Poorly fixed, inflamed, autolyzed or necrosed tissue samples, as well as tumors with sparse tissue, were excluded from the study sample. MonoclonalCD117and CD34 antibodies were reacted with sample tissue sections to determine mast cell density and microvessel density respectively.To minimize the possibility of bias, a histopathologist and an oral pathologist independently verified all findings.

Samples from the surgical specimens were collected and stored in labeled plastic containers containing formalin solution (10% buffered) for 24 to 36 hours. Tissue was then placed in an automatic tissue processor, Tissue Tek VIP-5 processor (Sakura, Japan). Slide processing involved dehydration with increasing strengths of alcohol, followed by clearing in which the alcohol was replaced by xylene at 38ºC. At 56ºC, the tissue was impregnated with melted paraffin wax. The Tissue Tek Embedding Console system (Sakura, Japan) was used to embed the tissue, followed by filling metal molds with new molten wax and allowing them to cool. Before being placed on slides, paraffin-embedded sections were trimmed and sliced into thin pieces of 4 microns using a microtome (Accu Cut Rotary Microtome SRM 200-1, Sakura, Japan). Finally, the sections were picked up from a water bath on sterile and marked frosted glass slides, then incubated for 3 hours at 60ºC and hematoxylin and eosin (H & E) stained with Varistain Multiproy slide strainer (Shandon, Germany). using a routine, validated procedure.Tumor grade, and immunohistochemistry was performed to quantify mast cell and microvessel density.

In the present study, indirect immunohistochemistry approach was used. It is a two-step technique in which an unlabeled primary antibody (first layer) reacts with tissue antigen before a secondary labeled antibody (second layer) reacts with the primary antibody. To perform immunohistochemistry, sections mounted on slides were de-paraffinized, re-hydrated, and a high temperature unmasking technique was employed. After that, sections were circled with a ImmEdge™ Hydrophobic Barrier PAP Pen before being washed in PBS (phosphate buffer saline) (pH 7.35 to 7.45) for 9 minutes (3 washes for 3 minutes each) and incubated with peroxidase blocker for 6 to 10 minutes, then washed with PBS for 9 minutes. In the first step, slides were incubated with primary antibody for 60 minutes, followed by washing in PBS buffer for 3–5 minutes. The second step comprised incubation of slides with an appropriate biotinylated secondary antibody and washing with PBS buffer. In the third step, slides were incubated with HRP (horse radish peroxidase) reagent followed by a PBS buffer wash. In the fourth step, slides were incubated in 3,3'-Diaminobenzidinel (DAB) buffer and DAB chromogen using dilution at a 1/0.1 ratio,

washed in distilled water,counterstained with hematoxylin, dehydrated, cleared, and mounted.

To determine MCD, tissue slices were reacted with a monoclonal CD117 antibody (Leica, Microsystems, Germany) according to the standard instructions. The Olympus BX53 microscope was utilized to examine the different antibody expression patterns on all tissue slices. Three microscopic high-power fields (HPFs) with maximal vascularity (hotspots) were located at low power (10x) for quantitative analysis of MCD, whereas the mast cell count was performed at higher magnification under an ocular grid (40x). An Olympus DP27 digital camera was used to take the images. The hotspots nearest to the invasive front of the tumor were selected. All mast cells that were immunoreactive to CD117 antibody were counted in a specific hotspot field, and the mean number of mast cells detected per HPF was reported for that special case. A brown mast cell granule cluster that was visibly isolated from the neighboring cell membrane was considered a single mast cell.

To determine MVD, tissue slices were reacted with a monoclonal CD34 antibody (Leica, Microsystems, Germany) according to the manufacturer's instructions. The number of microvessel in OSCC and NOM was counted using an Olympus BX53 microscope.The counts in four fields at a magnification of 40ximmunoreactive to CD34 antibodyon an ocular grid in the hotspot were counted, and the mean count in each case was recorded.Microvessels were detected by the presence of brown stained basement membrane surrounding them, whereas partly identifiable vessels were not counted. A single quantifiable microvessel was defined as any endothelial-lined vessel that appeared reddish brown and was clearly isolated from neighboring stromal and tumoral cells.The vessel lumen was not employed to define a microvessel; neither were red blood cells used to define vessel lumens.

Data entry was performed by research assistants and was cross-checked by other members for any potential errors. Where appropriate, descriptive statistics were presented as means, standard deviations, medians, interquartile range, and percentages. The Shapiro-Wilk and Kolmogorov-Smirnov tests were performed to determine the data set's normality. Pearson's correlation, ANOVA, and the student's t-test were used for statistical analysis. For statistical significance, a p-value of<0.05 and a 95% confidence interval were considered significant.

Results

A total of 40 cases were evaluated (20 NOM and WD OSCC each). There were 62.5% males and 37.5% females, with a mean (SD) age of 49.5 (18.1). In the OSCC group, the mean (SD) age was 57.3 (15.6) and 70% were male. The NOM group consisted of 55% men and 45% females, with a mean (SD) age of 41.8. (17.3).

Oral squamous cell carcinoma (OSCC) group:

The location of the biopsy varied between cases; 40% of the tissue samples were taken from the buccal mucosa, 30% from the tongue, 10% from the alveolus and lower lip each, and 5% each from the palate and retromolar area. Among cases, 25% were grade 1 (well differentiated) tumors, 45% were grade 2 (moderately differentiated) and 30% were grade 3 (poorly differentiated) tumors. The mean (SD) and median (IQR) MCD in OSCC were 22.4 (10.8) and 23.50 (20), respectively. Whereas, the mean (SD) and median (IQR) MVD in OSCC were 22.3 (7.48) and 22.50 (14.5), respectively. In OSCC, mast cell and MVD were shown to have a substantial, significant, negative connection meaning that with an increase in microvessel density, there was a significant decrease in mast cells. (r = -.702, p value =.001)**Figure 1.** It was also found that grade of tumor was positively correlated with microvessel density while negatively correlated with mast cell density. **Table-1** However, with the advancement of tumor grade, MCD was significantly reduced and MVD increased. **Table-2**

Table 1: Correlation between tumor grade, MCD and MVD in OSCC group

Variable	Grade of tumor	Mast cell density	Microvessel density
Grade of tumor	-	r = -.816*	r = .895*
Mast cell density	r = -.816*	-	r = -.702*
Microvessel density	r = .895*	r = -.702	-

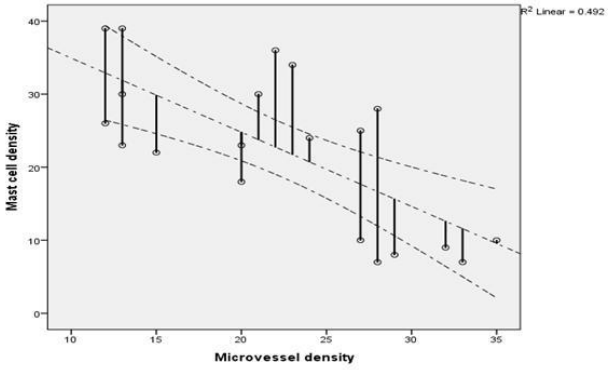
r = Pearson Correlation, * = significant at .001 (p-value

Table 2: Comparison of mean MCD and MVD in different grades of tumor

	N	Mean (SD)	Std. Error	df	F	p-value	95% CI for Mean	
							Lower	Upper
Mast Cell density								
Grade 1 tumor	5	31.2 (7.6)	3.42	17	28.6	.001	21.6	40.7
Grade 2 tumor	9	26.7 (5.7)	1.92				22.3	31.2

Grade 3 tumor	6	8.51 (1.3)	0.56				7.05	9.95
Microvessel Density								
Grade 1 tumor	5	13.0	1.22	17	34.3	.001	11.4	14.5
Grade 2 tumor	9	22.0	4.41				18.6	25.3
Grade 3 tumor	6	30.6	3.14				27.3	33.9

Figure 1: Correlation between MCD and MVD (spikes and 95% CI)



Normal oral mucosa (NOM) group:

In the NOM group, 30% of tissue samples were obtained from the buccal mucosa, 35% from the tongue, 10% from the alveolus, palate, and lower lip each, and 5% from the retromolar region. The mean (SD) and median (IQR) MCD in NOM were 22.4 (10.8) and 23.5 (20), respectively. The mean (SD) and median (IQR) MVD in NOM were found to be 11.5 (2.58) and 11 (3), respectively. There was no correlation found between MCD and MVD in NOM (r =.099, p-value =.677).

A noteworthy increase in the MCD was observed in the sections of OSCC when compared with NOM, signifying their role in the growth and advancement of tumors. However, as demonstrated in Table 2, when tumor grade increased, MCD reduced considerably. MCD was significantly higher (2–3 times) than NOM in tumor grades one and two of the OSCC, but when compared with grade three tumor the MCD was higher in NOM.MVD was significantly increased in OSCC sections compared to NOM sections. However, when tumor grade increased, MVD also increased significantly. Table 3 shows comparison of MCD and MVD in OSCC and NOM sections.

Table 3: Comparison of MCD and MVD in OSCC and NOM sections

	Number of Cases	**Mean (SD)**	**Std. Error of Mean**	**Median (IQR)**	**p-value**
Mast Cell Density					
OSCC	20	22.4 (10.8)	2.42	23.5 (20)	<.001
NOM	20	11.5 (2.58)	1.36	11 (3)	
Microvessel density					
OSCC	20	22.3 (7.48)	1.67	22.50 (14.5)	<.001
NOM	20	11.5 (2.58)	0.57	11 (3)	

Discussion

OSCC is one of the most common malignancies globally, with an annual global incidence of over 350,000 cases reported and 177,000 fatalities, albeit there are significant regional and environmental risk factor variances[15].Leading causes of mortality in OSCC patients are tumor infiltration, lymph node metastases, and high rates of recurrence[16]. Furthermore, late diagnosis is also one of the reasons behind the poor treatment outcomes, with a 5-year survival rate of less than 50% and recurrence after initial therapy and/or metastases is observed in more than half of the patients[17]. Although our insight into the molecular mechanisms involved in oncogenesis is advancing and many genetic markers have been proposed to greatly influence the diagnosis and prognosis of OSCC, no biomarker has yet fulfilled the standards essential for diagnostic and therapeutic application[1]

Mast cells are important among the numerous indicators found in OSCC, although their significance is yet unclear. Mast cells presence at the tumor-host tissue junction indicates that they play a significant role in host immune response. Mast cells potentially accumulate mediators that assist in immune defense, tissue disintegration, elastic change, and blood vessel formation[18] .Some researchers, however, conclude that the substances in tobacco-containing products can affect the presence, frequency, and stimulation of mast cells in oral cavity tissues[19]. The augmented mast cell densities in the tissue play a crucial role in modulating the cell machinery during tumorigenesis. The juxtaposition of mast cells to blood vessels promotes neo-angiogenesis, which is mediated by tryptase, heparin, TNF, bFGF, and TGF[20]]. It has been also reported that the extracellular matrix disintegration is favorably associated with mast cells, and they are implicated in the release and synthesis of matrix

metalloproteinases (MMPs) that can lead to the breakdown of collagen tissue [21]

Several studies have reported that mast cells exist in both normal and pathologic conditions and perform wide range of functions. According to Anuradha et al., mast cells play a significant role in the early stages of cancer progression with a significant increase in their number, whereas in later stages, their numbers decline as cancerous cells are no longer reliant on them for angiogenesis and can control neovascularization asynchronously[22]. A Norwegian study discovered that OSCC patients with low MCD had a worse prognosis and a reduced chance of survival, indicating that MCD might be utilized as an easy and practical tool for prognostic assessment[23].

Another study suggested that greater number of mast cells are associated with a better prognosis in OSCC. MCD decreases as tumor grade increases, emphasizing mast cells' protective role[24].Nakandala and colleagues found a substantial association between MCD and nodal metastasis, with both MCD and MVD being greater in OSCC with nodal metastasis[25]. Rajput and colleagues investigated breast tumors and discovered the existence of two factors: stem cell factor and c-kit, both are important in the functioning and proliferation of mast cells[26].) Increased mast cells have also been linked to poor prognosis in prostate and pancreatic cancer, melanomas, and leukemia, where they promote tumor growth[27]. In contrast, patients with sarcomas and lung adenocarcinomas showed a higher survival rate due to an increased number of mast cells, suggesting a favorable prognosis[28]. A study comparing normal oral mucosa to dysplasia and OSCC found an increase in the densities of microvessels and mast cells, which is in accordance with our findings. The enhanced angiogenesis in OSCC was believed to be caused by mast cells. If mast cells were solely responsible for neovascularization, they would have increased rather than decreased as tumor grade worsened, indicating the presence of other mediators influencing angiogenesis[29]. According to the conclusions of various studies, mast cells may have a protective or destructive role in different types of malignancies, and it is vital to investigate each cancer individually to adequately understand the role of mast cells.

Conclusion

Our findings may help to develop a criterion for indicating disease progression in oral malignancies as well as assist in understanding emerging therapeutic approaches such as mast cell degranulation blockage agents. Moreover, the relationship of MVD with tumor grade implies that it might be utilized as an additional parameter to histologically grade malignancies as well as provide an empirical evaluation for treatment modalities.

Limitations and Future Directions

Our study's small sample size may limit the generaliz ability of conclusions. Moreover, we only investigated mast cells and angiogenesis in the present study. It is crucial to explore other biomarkers implicated in oncogenesis to better understand the unique role of each marker.

In initial stages, mast cell buildup might be associated with increased neovascularization, tumor aggressiveness, and metastatic spread. Therefore, these cells might be a potential target for tumor adjuvant therapy. This can be achieved through the selective inhibition of angiogenesis. Targeting several biomarkers in future large-scale investigations will be critical in establishing the precise involvement of mast cells in oncogenesis.

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