



IMMUNOHISTOCHEMICAL EVALUATION OF ANGIOGENESIS BY CD34 AND MAST CELL DENSITY IN DIFFERENT GRADES OF ORAL AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

Pathology

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ABSTRACT

Aims and Objective: To compare mast cell density and microvessel density in different grades of oral and oropharyngeal squamous cell carcinoma in comparison to normal oral mucosa.

Methods: A study was done in the Department of Pathology, MAMC Agroha. 40 consecutive cases of different grades of oral and oropharyngeal squamous cell carcinoma along with normal oral mucosa from February 2018 to March 2019 were included in the study. Biopsies were fixed in 10% formalin and histopathological slides were prepared for staining with haematoxylin and eosin, toluidine blue and immunohistochemical staining using CD 34 immunomarker.

Results: Micro vessel density and Mast cell density were found to be increased from normal oral mucosa to different grades of oral and oropharyngeal squamous cell carcinoma and a positive correlation was found between microvessel density and mast cell density but was not statistically significant.

Conclusion: Our present study concluded a positive correlation between microvessel density and mast cell density reaching to idea that mast cells are one of the important factor for tumour progression via promotion and upregulation of angiogenesis, although the absence of statistical significance suggests the remarkable role of other angiogenic and non angiogenic factors in tumour progression.

KEYWORDS

Squamous cell Carcinoma, Angiogenesis, Mast cells.

INTRODUCTION

Carcinoma of oral cavity and oropharynx are the most common cancer worldwide.¹ In India oral and oropharyngeal carcinoma accounts for 30% of all carcinoma.² The incident cases of oral cavity and oropharyngeal carcinoma in India in 2016 was 113,000 and 65,000 respectively.³ In India the incidence of oral and oropharyngeal carcinoma will increase to more than 1.7 million in 2035 indicating that death rate will also increase from 68000 to 1-2 million in same period as predicted by International agency for research on cancer.⁴

Oral and oropharyngeal squamous cell carcinoma has a complex biological behaviour and despite the advances in the treatment modalities, 5-year survival rates have not improved more. Thus leading to an interest for predicting the possible future behaviour, so that alternative therapeutic strategies can be implemented for treatment of carcinoma.⁵

Carcinoma spreads to adjacent and distant organs either by hematogenous or lymphatic route. Vascular network forms an important source of metastasis and requires new blood vessel formation known as angiogenesis.⁶

Angiogenesis being a critical process depends on the positive and negative angiogenic mediators within the vascular microenvironment.⁷ The tumor microenvironment is an interactive, organized, and dynamic environment where cancer cells as well as many different cellular and biochemical structures coexist and continuously interact with each another.⁸

Angiogenesis is influenced by many cell types including endothelial cells and their precursors, smooth muscle cells, fibroblasts, neutrophils, eosinophils, basophils, and mast cells.⁷ Among these, mast cells have been propounded as promoter for angiogenesis.⁹ Mast cells can either act like pro-tumorigenic or antitumorigenic molecule depending on the milieu. In several solid tumors like thyroid, gastric, pancreas, bladder cancers and merkel cell carcinoma, mast cells always appeared as pro-tumorigenic. On the contrary in breast carcinoma mast cells played antitumorigenic role.¹⁰

Increased numbers of mast cells are found in pancreatic adenocarcinomas, squamous cell carcinomas of the esophagus, mouth, and lip. Increased numbers of mast cells have been associated with

unfavorable disease outcomes in some tumors on the other side of coin increased number also correlated with good prognosis.¹¹

Clinical and experimental evidence has demonstrated that new vessel formation is essential for tumor growth and progression and hence represents the overall aggressiveness and the prognosis of tumor.¹² Some laboratory investigations have documented a decreased rate of tumor angiogenesis in mast cell deficient mice, as to human pathology, an increased number of mast cells has been reported in angiogenesis associated with a number of vascular, solid and hematological neoplasms.¹³ An association between mast cell and new vessel formation has also been reported in stomach, esophagus and oral cavity.¹⁴

Only few studies have been done to evaluate association between mast cell density and microvessel density in different grades of oral and oropharyngeal squamous cell carcinoma, out of which some directly correlate them while others show controversial results and doesn't reach to any inference. Thus our present study was planned and executed with intension to evaluate the role of mast cells and angiogenesis in different grades of oral and oropharyngeal squamous cell carcinoma along with comparison to normal oral mucosa.

AIM AND OBJECTIVES

1. To demonstrate mast cell density using Toluidine blue stain.
2. To demonstrate intratumoral microvessel density using CD34 immunomarker staining.
3. To compare mast cell density and microvessel density in different histological grades of squamous cell carcinoma of oral cavity and oropharynx in comparison to normal oral mucosa.

MATERIAL AND METHODS

A retrospective study conducted in the Department of Pathology, Maharaja Agrasen Medical College, Agroha (Hisar) and comprised of 40 cases of different grades of oral and oropharyngeal squamous cell carcinoma along with normal oral mucosa from February 2018 to March 2019 were included in the study. Out of these 4 cases of normal oral mucosa and among the carcinoma cases 13 well differentiated, 18 moderately differentiated and 5 poorly differentiated, cases were included.

The relevant clinical history and clinical profile of the patient including age, gender, anatomic location, status of tobacco and alcohol intake were recorded.

SPECIMEN PREPARATION:

The biopsy specimen were grossly examined and fixed. Then formalin fixed paraffin embedded tissue were sectioned at 4 μm and routine Haematoxylin and Eosin staining done and cases with histological diagnosis of squamous cell carcinoma were graded into well, moderate and poorly differentiated using histological malignancy grading system by Bryne and further submitted to immunohistochemical staining using Monoclonal Mouse Anti-Human CD 34 immunomarker for intratumoral microvessel density and toluidine blue staining for mast cell density.

Toluidine blue staining

The slides were flooded with working toluidine blue solution for 1–2 min and then immediately were rinsed in distilled water.

Results of staining:

Mast cells Interpretation

Granules- Purple or bright magenta.

Nucleus- Blue

Background- Blue

Mast cells were identified as clusters of granules appearing purplish with light blue

background and clearly separate from adjacent clusters were considered as single mast cell.

Mast cell density was calculated from the 4 fields using the formula: $MCD (cells/mm^2) = \text{Number of mast cells in a field} / \text{Area of a field}$.

Immunohistochemical Staining

Sections were cut to 3–4μm thickness and mounted on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in alcohol. Antigen retrieval was done using Tris EDTA buffer at PH 9 in a pressure cooker for 20 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxidase. Application of primary antibody Monoclonal Mouse Anti-Human CD34 Class II ready to use (DAKO) for 40 minutes was done followed by washing in Tris Buffer Saline. After that incubation with polymerized horseradish peroxidase (HRP)-anti-mouse/rabbit immunoglobulin IgG (Secondary antibody) (DAKO) for 30 minutes was done. Then the sections were incubated in DAB reagent (3, 3diamino benzidinetri hydrochloride) solution for 10 minutes. Then the sections were counterstained with Mayer's hematoxylin.

Interpretation: Vascular Endothelial cells: Brown Microvessel density (MVD) was assessed by light microscopy in intratumoral areas with highest number of blood vessels (hot spots) according to the method described by Weidner et al.¹⁵ Any endothelial-lined vessel lumen or endothelial cell cluster appearing reddish brown and clearly separate from adjacent clusters were considered as a single countable microvessel.

Microvessel density was calculated from each 4 fields using the formula.

$MVD (vessel/mm^2) = \text{Number of blood vessels in a field} / \text{Area of field}$

Statistical Analysis-

The data was entered in Microsoft excel spread sheet and then analysed by using SPSS 20.0. Data is presented as frequency and percentages wherever applicable. Post Hoc using Bonferroni procedure test and Pearson's correlation was used to compare descriptive variables. P-value ≤ 0.05 was considered statistically significant.

OBSERVATION AND RESULTS

Out of 40 cases, 34 (85%) cases were male and rest were females 6 (15%). Minimum and maximum ages of study subjects were observed as 18 years and 85 years respectively with mean age as 55.35 years and standard deviation as 15.31 years. Risk factors evaluated for the study were tobacco and alcohol intake and were present in 70% and 30% of the patients respectively. All 40 cases were distributed into study groups and categorized as 4 (10%) normal oral mucosa, 13 (32.5%) well differentiated, 18 (45.0%) moderately differentiated and 5 (13%) poorly differentiated squamous cell carcinoma.

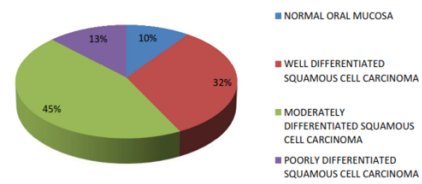


Figure 1 Showing distribution of cases into study groups

In the present study of 40 cases, 36 cases of oral and oropharyngeal squamous cell carcinoma were categorized on the basis of location of the lesion except those 4 cases of normal oral mucosa. Out of 36 cases, maximum number of cases 14 (39%) were located on tongue, followed by 10 (28%) cases from buccal mucosa. Rest of the cases included tonsil 5 (14%), valleculae 4 (11%), soft palate 3 (8%).

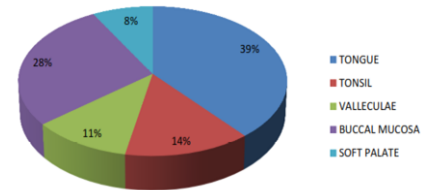


Figure 2 Showing distribution of squamous cell carcinoma cases according to their location (n=36)

Analysis of mean microvessel density:

The mean microvessel density in normal oral mucosa, well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma were found as 60.48±16.30, 104.97±29.46, 147.95±44.29 and 213.25±78.26 respectively. The mean microvessel density was analysed using ANOVA test and the mean was found to be higher with disease progression and also in comparison to normal oral mucosa. This difference in mean microvessel density was found to be statistically significant, (p < 0.05).

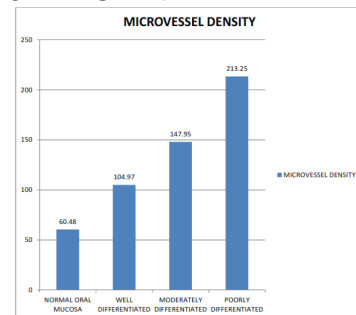


Figure 3 Showing mean microvessel density among study groups

Analysis of mean mast cell density:

The mean mast cell density in normal oral mucosa, well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma were 10.63±4.65, 44.95±9.44, 53.91±17.40 and 54.51±45.11 respectively and the mean mast cell density was found to be higher with progressive grades and also in comparison to normal oral mucosa and this difference was statistically significant, (p < 0.05).

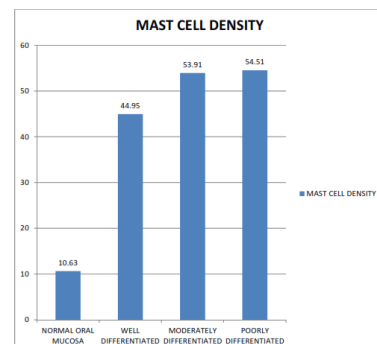


Figure 4 Showing mean mast cell density among study groups

Correlation between microvessel density and mast cell density:

For evaluating correlation between microvessel density and mast cell density Pearson Correlation was applied

		MICROVESSEL DENSITY	MAST CELL DENSITY
MVD	Pearson Correlation	1	0.303
	Significance (2-tailed)		0.058
	N	40	40
MCD	Pearson Correlation	0.303	1
	Significance (2-Tailed)	0.058	
	N	40	40

In this study a positive correlation was found between Microvessel density and Mast cell density ($r=0.303$) but this correlation was not found to be statistically significant.

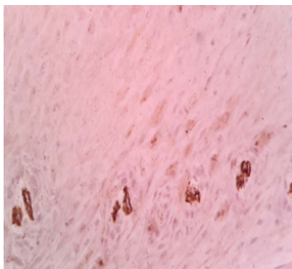


Figure 5 Showing expression of CD34 positive microvessels in normal oral mucosa (40x)

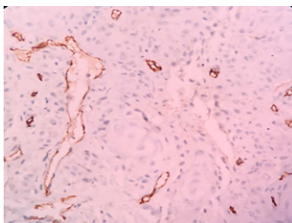


Figure 6 Showing expression of CD34 positive microvessels in WDSCC (40x)

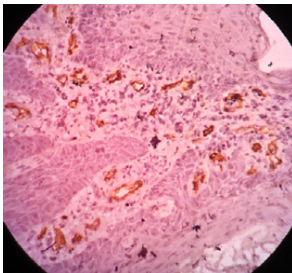


Figure 7 Showing expression of CD34 positive microvessels in MDSCC (40x)

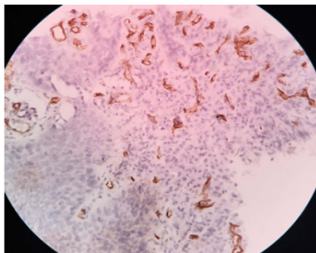


Figure 8 Showing expression of CD34 positive microvessels in PDSCC (40x)

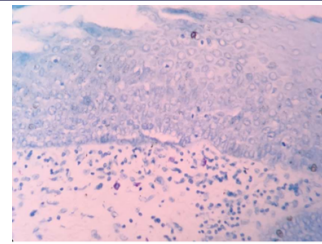


Figure 9 Showing mast cells positive for toluidine blue in NOM (40x)

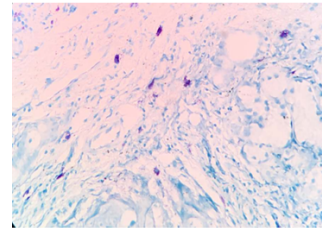


Figure 10 Showing mast cells positive for toluidine blue in WDSCC (40x)

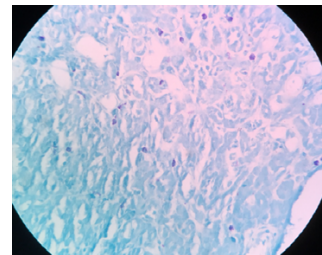


Figure 11 Showing mast cells positive for toluidine blue in MDSCC (40x)

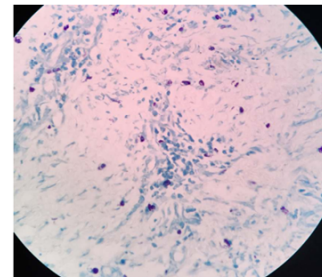


Figure 12 Showing mast cells positive for toluidine blue in PDSCC (40x)

DISCUSSION

Only few studies have been done to evaluate association between mast cell density and microvessel density in different grades of oral and oropharyngeal squamous cell carcinoma and some studies have controversial results. Therefore in our study we evaluated mast cell density and microvessel density in different grades of oral and oropharyngeal squamous cell carcinoma along with in comparison to normal oral mucosa.

Analysis of mean microvessel density:

In the present study mean microvessel density in normal oral mucosa, well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma were 60.4825 ± 16.30 , 104.9769 ± 29.46 , 147.95 ± 44.29 and 213.25 ± 78.26 respectively. Mean MVD was found to be significantly increased from normal oral mucosa to grade progression i.e. well, moderately and poorly differentiated oral and oropharyngeal squamous cell carcinoma and in comparison to normal oral mucosa. ($p < 0.05$)

The result of our study was in concordance with the study done by and Kathuriya et al¹⁶ they showed that microvessel density significantly increased in different grades of oral squamous cell carcinoma as compared to normal oral mucosa.

Supporting this observation Kalra et al¹⁷ assessed the microvessel density by and draw the similar inference.

In 2017, Kabiraj et al¹⁸ depicted a significant increase in microvessel density in oral squamous cell carcinoma when compared the normal oral mucosa.

These all studies also concluded that intratumoral blood vessels are known to play an important role in cancer growth by supplying oxygen, nutrients and excreting metabolic products.

Analysis of mean mast cell density:

The mean mast cell density in normal oral mucosa, well, moderately and poorly differentiated squamous cell carcinoma were 10.63±4.65, 44.95±9.44, 53.91±17.40 and 54.51±45.11 respectively. The mean mast cell density was also observed to be higher with progression of grade and as compared to normal oral mucosa and this difference was statistically significant, ($p < 0.05$).

The result of our study was in concordance with the study done by Jahanshahi et al¹⁹ in which they concluded a significantly higher mast cell density in oral squamous cell carcinoma compared to normal oral mucosa.

Corresponding to our study Ramsridhar et al²⁰ carried out a study on mast cell density in 50 biopsy specimens and found that mast cells were significantly higher in oral squamous cell carcinoma compared to normal oral mucosa.

But our results are in contrast to studies by Belgaumi et al²¹ and Phillip Brockmeyer et al²² who reported a decrease in the number of mast cell as the grade of tumour progressed from well to poorly differentiated oral squamous cell carcinoma suggesting a protective role of mast cells in oral squamous cell carcinoma.

Correlation between microvessel density and mast cell density:

For evaluating correlation between microvessel density and mast cell density Pearson Correlation was applied, a positive correlation was found between them ($r=0.303$) illustrating that mast cells play a key role in tumour angiogenesis. However the result was not statistically significant ($p > 0.05$), indirectly suggesting the role of other factors too in angiogenesis and tumor progression.

This observation correlates with a study done by Tahir et al²³ where he illustrated that mean MVD and Mean MCD were insignificantly correlated with each other despite their significant rise in oral squamous cell carcinoma.

Nakandala et al²⁴ also reported similar finding according to their analysis there was a positive correlation between mast cell density and microvessel density but this was not statistically significant, ($p > 0.05$). Based on the findings of our study it is deduced that mast cell density and microvessel density increases from normal oral mucosa to progressive grades of oral and oropharyngeal squamous cell carcinoma.

CONCLUSION

It is deduced in our study that mast cell density and microvessel density increases from normal oral mucosa to progressive grades of oral and oropharyngeal squamous cell carcinoma. Our present study also concluded a positive correlation between mean vascular density and mean mast cell density in normal and different histological grades of oral and oropharyngeal squamous cell carcinoma, thus reaching to agreement with idea of considering mast cell as one of the important factor for tumour progression via promotion and upregulation of angiogenesis.

Though mast cell plays cardinal role in angiogenesis yet the absence of statistical significance suggests the remarkable role of other angiogenic and non angiogenic factors in tumour progression and clinical behaviour of it.

However, looking at the influential role of angiogenesis in the evolution of portentous oral and oropharyngeal lesions, large scale multi-institutional studies are required on different grades of squamous cell carcinoma to investigate contribution of mast cells and other angiogenic factors in planning and development of various adjuvant therapeutic strategies like anti angiogenic therapy in cancer.

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