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Original Article

Pathology Section

Tumour Associated Tissue Eosinophilia in Oral Squamous Cell Carcinoma by Special Histochemical Analysis of Tissue Eosinophilia using Congo Red Staining

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ABSTRACT

Introduction: Tumour Associated Tissue Eosinophilia (TATE) is an important phenomenon occurring in the tumour microenvironment and has a pivotal role. TATE is defined as "eosinophilic stromal infiltration of a tumour not associated with tumour necrosis or ulceration". The exact role of TATE in malignancies is yet unclear but studies have shown that TATE usually has a favourable outcome in head and neck Squamous Cell Carcinoma (SCC). Therefore, more studies are needed to substantiate this data.

Aim: To compare TATE between normal epithelium and Oral Squamous Cell Carcinoma (OSCC) and compare TATE between the histological grades of OSCC.

Materials and Methods: A retrospective study was conducted at Dr. BR Ambedkar Medical College, Bangalore, Karnataka, India, from June 2019-January 2021 which included 50 cases, 10 from normal mucosa and 40 histopathologically diagnosed cases of OSCC. 1% congo red solution was used to stain 4 µm thick sections. The sections were examined at a high magnification (40X) and 10 microscopic fields were examined in succession for eosinophils. The average number of eosinophils observed were compared using univariate analysis, which included the Analysis of Variance (ANOVA) test and the Chi-square test and a p-value of 0.001 was considered statistically significant.

Results: The mean value of tissue eosinophils increased in OSCC compared to normal mucosa, according to present study findings. When comparing different grades of carcinoma, statistical analysis revealed that well differentiated squamous cell carcinoma had a greater TATE than other grades, which was statistically significant (p-value <0.001).

Conclusion: The higher eosinophil count in well differentiated OSCC compared to the other grades could be associated with a better clinical outcome for the patient. In OSCC, TATE can be utilised as a predictor of a good prognosis.

Keywords: Eosinophils, Eosinophillic stromal infilteration, Intratumoural inflammatory infiltrate, Special stains

INTRODUCTION

The OSCC is "carcinoma with squamous differentiation arising from the mucosal epithelium" [1]. The OSCC is the sixth most frequently occurring cancer in the world, accounting for more than 90% of all oral malignancies. In most developing countries, they have become a major health issue. OSCC's account for over 30% of all new cases and 22.9% of cancer-related fatalities in India [2].

The TATE is defined as "eosinophilic stromal infiltration of a tumour not associated with tumour necrosis or ulceration" [3]. The presence of eosinophils as a component of the peritumoural and intratumoural inflammatory infiltrate characterises the tumour microenvironment [4].

The TATE has been studied in malignant neoplasms, particularly in head and neck tumours. TATE's application as a potential prognostic indicator has been investigated, with mixed results. The present study was aimed to quantify and compare TATE in normal mucosa and in the inflammatory infiltrate in different histopathological grades of OSCC using Congo red staining.

MATERIALS AND METHODS

The present retrospective study was conducted in the Department of Pathology, Dr. BR Ambedkar Medical College, Bengaluru, Karnataka, India, from June 2019-January 2021 and the analysis of the study was done from February-July 2021 on normal oral mucosa and OSCC tissue sections to compare the TATE between normal epithelium and OSCC. TATE was also compared with the different grades of OSCC.

The study included 50 tissue samples, 40 samples were histopathologically diagnosed cases of OSCC, out of which 22 cases belonged to well differentiated OSCC, 12 cases belonged to moderately differentiated OSCC and six cases were of poorly differentiated OSCC with 10 cases of normal mucosa that were included in this study.

Inclusion criteria: All oral biopsies and radical resection specimens from patients with OSCC of age more than 18 years were included in the study.

Exclusion criteria: Primary malignancies of the oral cavity other than OSCC recurrence and metastatic tumours of the oral cavity cases with extensive tumour necrosis without sufficient viable tissue for accurate evaluation were excluded in the study.

Study Procedure

A 5 µm thick sections were made using Leica RM 2125 and stained using Congo red special stain. The steps involved were-

- Deparaffinize sections
- Hydrate sections through graded levels of alcohol to water.
- The sections were stained with 1% Congo red solution for 20 minutes
- Sections washed with water.
- Differentiating quickly (5-10 dips) in Alcoholic Sodium hydroxide solution and then washing in tap water for 1 minute.
- Counterstaining with haematoxylin was done for 30 seconds and then slides were rinsed in tap water for 2 minutes.

- Dehydration, clearing and mounting with DPX (dibutyl phthalate in xylene) mounting media was done.

Interpretation of congo red staining: The eosinophils were counted in 10 consecutive High-Power Fields (HPF) at 40x magnification, and an absolute number was assigned. It was then subdivided into the groupings 1-10, 11-50, 51-100, 101-200, and >200. The absolute count did not include necrotic sites with eosinophilic infiltration.

STATISTICAL ANALYSIS

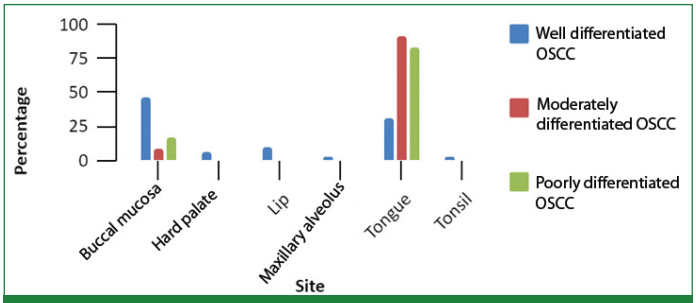
Continuous measurement results were displayed as Mean±Standard Deviation (SD) (min-max), while categorical measurement results were presented as Number (%). The chi-square test, a non parametric setting for qualitative data analysis, was used to determine the significance of research parameters on a categorical scale between two or more groups. A statistically significant value of p<0.001 was used. Univariate analysis utilising the ANOVA test and the Chi-square test was used to compare the average number of eosinophils obtained in different grades of OSCC.

RESULTS

The study was conducted on 50 samples (40 cases and 10 normal mucosa). The predominant age group which presented with OSCC was in fourth decade accounting for 32% with a male preponderance [Table/Fig-1]. The most common site for OSCC was the lateral border of the tongue but among the well differentiated OSCC, the most common location was the buccal mucosa [Table/Fig-2,3].

Age (years)	Gender		Total, n (%)
	Female, n (%)	Male, n (%)	
<40	2 (11.1)	6 (18.8)	8 (16)
41-50	3 (16.7)	13 (40.6)	16 (32)
51-60	5 (27.8)	5 (15.6)	10 (20)
61-70	6 (33.3)	6 (18.8)	12 (24)
>70	2 (11.1)	2 (6.3)	4 (8)
Total	18 (100)	32 (100)	50 (100)

[Table/Fig-1]: Age and gender frequency distribution of patients studied.
P=0.297; Not Significant; Fisher exact test

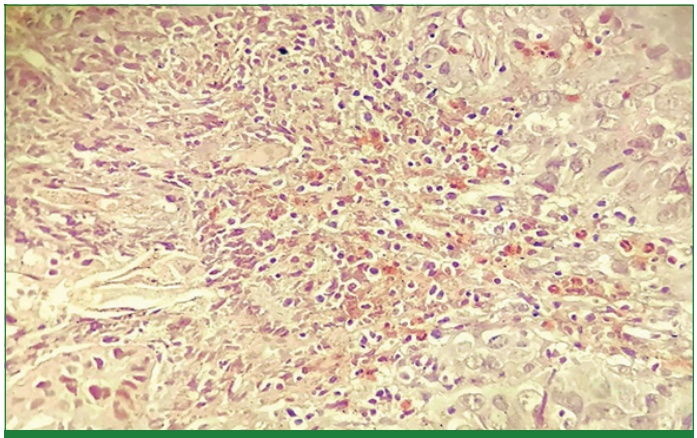


[Table/Fig-2]: Site-distribution in relation to different grades of OSCC.

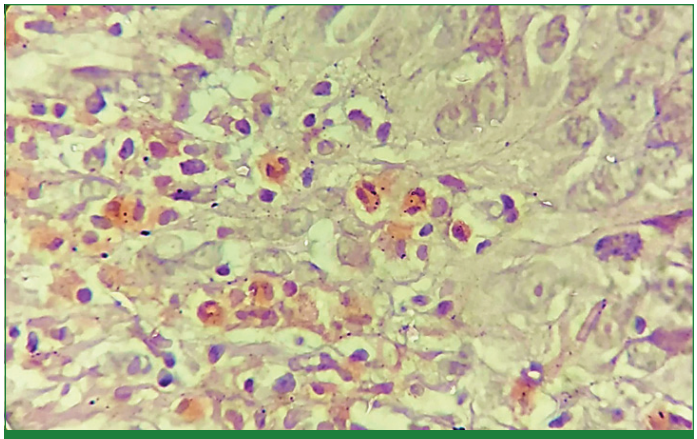
Site	Histopathological grading				Total n (%)
	Well differentiated OSCC n (%)	Mod differentiated OSCC n (%)	Poorly differentiated OSCC n (%)	Normal mucosa n (%)	
Buccal mucosa	10 (45.5)	1 (8.3)	1 (16.7)	5 (50)	17 (34)
Hard palate	2 (9.1)	0	0	0	2 (4)
Lip	2 (9.1)	0	0	1 (10)	3 (6)
Maxillary alveolus	1 (4.5)	0	0	0	1 (2)
Tongue	6 (27.3)	11 (91.7)	5 (83.3)	4 (40)	26 (52)
Tonsil	1 (4.5)	0	0	0	1 (2)
Total	22 (100)	12 (100)	6 (100)	10 (100)	50 (100)

[Table/Fig-3]: Site-distribution in relation to histopathological diagnosis of patients studied.
P=0.073+, Significant, Fisher Exact Test

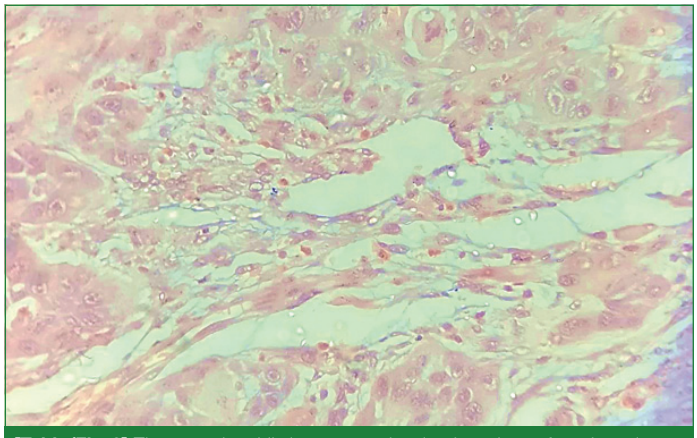
Normal mucosa showed predominantly 1-10 eosinophils/10 HPF which was significantly lower when compared to OSCC. Among the different grades of OSCC, the average eosinophil count per 10 HPF was higher in well differentiated OSCC [Table/Fig-4,5] as compared to moderately differentiated [Table/Fig-6] and poorly differentiated OSCC [Table/Fig-7]. The mean eosinophils were highest in well differentiated OSCC (151.64±87.59] and least in normal mucosa (2.20±2.70]. This comparison was statistically significant (p-value <0.001); [Table/Fig-8]. Tissue eosinophils showed significant association with grade of tumour [Table/Fig-9] F=3.185, p<0.001: ANOVA test).



[Table/Fig-4]: Tissue eosinophils in congo red stained sections of well differentiated OSCC (100X magnification).



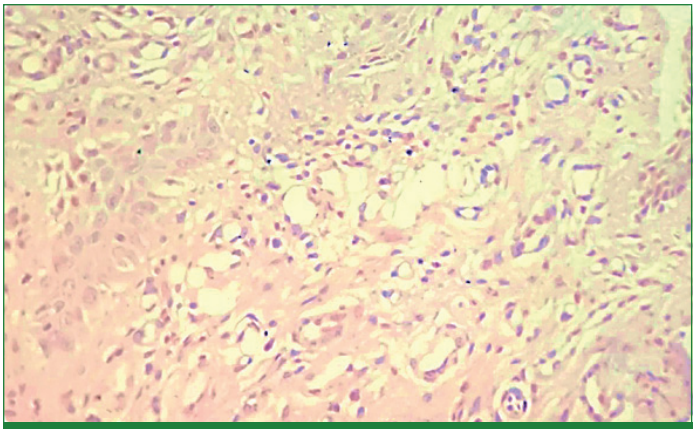
[Table/Fig-5]: Tissue eosinophils in congo red stained sections of well differentiated OSCC (400X magnification).



[Table/Fig-6]: Tissue eosinophils in congo red stained sections of moderately differentiated OSCC (400X magnification).

DISCUSSION

The OSCC is a carcinoma with squamous differentiation arising from the mucosal epithelium. It is an epithelial neoplasm that starts as a localised overgrowth of mutated stem cells near the basement membrane and spreads upward and laterally, eventually obliterating the normal epithelium. OSCC is the world's sixth most prevalent



[Table/Fig-7]: Tissue eosinophils in congo red stained sections of poorly differentiated OSCC (400X magnification).

Eosinophils/ 10 HPF	Histopathological grading				Total
	Well differentiated OSCC	Mod differentiated OSCC	Poorly differentiated OSCC	Normal mucosa	
0	0	0	0	4 (40.0%)	4 (8.0%)
1-10	2 (9.1%)	1 (8.3%)	0	6 (60%)	9 (18%)
11-50	2 (9.1%)	5 (41.7%)	6 (100%)	0	13 (26%)
51-100	4 (18.2%)	5 (41.7%)	0	0	9 (18%)
101-200	7 (31.8%)	1 (8.3%)	0	0	8 (16%)
>200	7 (31.8%)	0	0	0	7 (14%)
Total	22 (100%)	12 (100%)	6 (100%)	10 (100%)	50 (100%)

[Table/Fig-8]: Eosinophils/10HPF- distribution in relation to histopathological diagnosis of patients studied.
p≤0.001**; Significant; Chi-square test

Histopathological grading	N	Mean	Standard deviation	Standard error	ANOVA	p-value
Well differentiated OSCC	22.00	151.64	87.59	18.67		
Mod differentiated OSCC	12.00	54.58	28.07	8.10	52.38	<0.001**
Poorly differentiated OSCC	6.00	29.00	12.88	5.26	26.80	<0.001**
Normal mucosa	10.00	2.20	2.70	0.85	-	-
Total	50.00	83.74	86.52	12.24	17.617	<0.001**

[Table/Fig-9]: Comparison of tissue eosinophils according to histopathological diagnosis of patients studied.
Tissue eosinophils is significantly linearly correlated with histopathological diagnosis with F=17.617, p<0.001

malignancy. OSCC account for more than 90% of malignancies in the mouth [1]. An estimate of around 377,713 new cases of OSCC in 2020 were made by the Global Cancer Observatory: Cancer Today (GLOBOCAN) project with a global age-standardised incidence rate of 4.1 cases per 100,000 people and a global mortality rate of 1.9 fatalities per 100,000 people [5].

Oral cancer is the most common type of cancer in India, accounting for up to 40% of all cancers. OSCC accounts for the majority of oral cancer cases, with incidence rates of 12.8 and 7.5 per 100,000 in men and women, respectively, with the majority of patients aged 40 to 70 years [6]. Present study also revealed a male preponderance, with the majority of cases occurring in the fourth decade.

It is the most common cancer in men and the third most common cancer in women, and it is linked to harmful oral habits such as tobacco chewing, betel quid chewing, tobacco smoking, and reverse smoking, as well as other factors such as alcohol consumption, low socio-economic status, poor hygiene, poor diet, viral infections, chronic irritation from ill-fitting dentures, rough or fractured teeth. Any part of the oral mucosa might be affected by OSCC. The tongue, floor of the mouth, and gingiva are the most common sites in many populations, while OSCC commonly affects the buccal mucosa in Asian populations due to tobacco and betel quid chewing [1]. In betel quid chewers, carcinoma of the buccal mucosa and lateral tongue is prevalent because the quid is pushed against the buccal mucosa. In India, betel quid chewers are a high-risk population, and the most common cancer in the community is carcinoma of the buccal mucosa and lateral tongue. Present study also showed tongue to be the most common site for OSCC (52%) followed by buccal mucosa (34%).

Cancer and its invasiveness are influenced by the tumour microenvironment as well as genetic changes. TATE, according to Leighton SEJ et al., is defined as eosinophil infiltration of the tumour without the evidence of necrosis or ulceration [3]. In head and neck tumours, intense TATE ranges from >10 to 100 eosinophils per HPF, according to the literature [7]. Absolute Eosinophilia is defined as eosinophils >450 per cubic millimetre [8]. The biological interactions that occur between cancer cells, immune effectors, and inflammatory cells, as well as cells from the tumour vasculature and stroma, are referred to as the "tumour microenvironment." [9].

Host immune response cells such as CD8+ T-cells, macrophages, CD4+ T-cells, and eosinophils promote cancer biology evolution. The exact role of eosinophils in malignancies is uncertain, they have been related to the production of cytotoxic proteins. The most important protein implicated is the eosinophil cationic protein, a single polypeptide chain with a molecular mass ranging from 15-22 kDa encoded by the Ribonuclease A Family Member 3 (RNSE3) gene on chromosome 14q11.2 [10].

Few parts of the tumour develop hypoxic/tissue necrosis as it grows. IL-5, IL-3, eotaxin-1, as well as thymus and activation-regulated chemokines, are all produced by necrotic tumour cells (TARC or CCL17). These variables, in combination with other stimuli, cause tissue eosinophilia by triggering eosinophil differentiation and/or migration from circulation [11]. According to Lorena SC et al., the predominant source of eotaxin in OSCC is eosinophils, indicating an autocrine pathway for tissue eosinophilia [12].

Various stromal responses have been connected to invasive carcinomas. Inflammatory cell infiltration, particularly mononuclear cells like lymphocytes and plasma cells, as well as desmoplastic fibroblast development, are the most common responses. Inflammatory cells usually contain eosinophilic leukocytes. Invasive carcinomas of the uterine cervix have cellular responses that include some eosinophils in one-fourth to two-thirds of cases. In 4.4-13.65% of infiltrative cervical carcinomas, eosinophil-dominated stromal responses have been observed [13].

In the present study, the overall age of OSCC patients ranged between 41-50 years with male predominance. In this study, the most common grade of OSCC was well differentiated. Studies by Debta P et al., Lowe and Fletcher CD., Gold Smith MM et al., Kargar et al., Dorta RG et al., and Halimi J et al., [7,14-18]. found that TATE was seen greater in OSCC as compared to the normal mucosa and was found to be more in well differentiated OSCC which correlated with the results obtained in this study. Increased quantities of tissue eosinophils have also been linked to an anti-tumour effect and consequently to a positive prognosis, according to their research [Table/Fig-10] [14,17,19].

Variable	Shrestha P et al., [19]	Kargar R et al., [17]	Debta P et al., [14]	Present study
Mean tate (OSCC)	233.5	114.69	71.89	151.64
Mean tate (normal mucosa)	1	15.71	1.90	2.20
p-value	0.002	<0.001	<0.05	<0.001

[Table/Fig-10]: Comparison between mean TATE in normal mucosa and OSCC with other studies [14,17,19].

Studies like Hu G et al., suggest that TATE is possibly able to regulate the tissue homeostasis of the tumour microenvironment and inhibit tumour growth and metastasis thereby improving survival [20].

Hence, there are several studies that consider TATE as a favorable prognosis. One must also acknowledge that there are also studies that have shown TATE to be associated with poorer prognosis like Sassler AM et al., and Leighton SEJ et al., and some even consider TATE to have no influence on the clinical outcome of the patient like the study by Oliveira et al. All these findings indicate that TATE and its importance is still a matter of research and controversy [3,21,22].

Limitation(s)

The sample size of the study was small and being a retrospective study, follow-up of patients was not possible to know the outcome of the disease. A wide scale analysis with follow-up studies can provide more insight and TATE could become a routine prognostic marker in Oral squamous cell carcinoma.

CONCLUSION(S)

The present study shows a significant association between TATE and OSCC. High TATE in well differentiated OSCC could be associated with good prognosis and anti-tumoural properties of OSCC which was substantiated by several other studies which showed similar results. Even though eosinophils can be identified with relative ease in routine H&E-stained sections of tissue, using special stains like Congo red can help highlight the eosinophils for better identification and counting. Hence, histochemical analysis of TATE using Congo red in OSCC is an inexpensive tool which will help pathologists in evaluating the prognosis of patients. This can help in better follow-up of patients.

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