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Quantification analysis of tissue eosinophilia in squamous cell carcinoma of the head and neck region



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ABSTRACT

Background and aim: Tumor-associated tissue eosinophilia (TATE) is commonly present in different neoplasms, yet its relationship with cancers has not been established. Squamous cell carcinoma (SCC) is one of the most common malignancies of the head and neck region. The aim of this study was to evaluate the relationship between TATE and histological grading of head and neck SCC (HNSCC) using Luna staining technique.

Materials and methods: In the present descriptive study, 67 samples consisting of 4- μ m cross-sections of HNSCC tissue stained with hematoxylin and eosin (H&E) were re-evaluated for histological grading. The samples were divided into three categories of well differentiated, moderately differentiated, and poorly differentiated. Luna histochemical staining was used to evaluate infiltration of eosinophils in the tumor. Eosinophils were randomly counted in 10

microscopic fields and divided into 4 categories. Kruskal-Wallis test and Spearman's correlation coefficient were used to evaluate the relationship between eosinophilic infiltration and other relevant factors.

Results: The frequency of tissue eosinophilia was zero in only one case (1.5%) and was positive in the rest 66 cases (98.5%). The mean count of eosinophils was 7.5 ± 4.5 cells per high-power field (HPF). There was a significant relationship between histopathological grading and TATE ($p = 0.04$). However, there were no significant relationships between TATE and factors such as age, gender, and lesion location.

Conclusion: The tissue eosinophil counts in the group with poorly differentiated SCC of the head and neck region were less than those in the two other groups; regardless, no significant relationship was found with other clinicopathological factors.

Keywords: Eosinophils, squamous cell carcinoma, head and neck, Luna staining

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INTRODUCTION

Squamous cell carcinoma (SCC) is the most common malignancy of the head and neck region and the oral cavity, particularly originating from the stratified squamous epithelium of the mucosa. It accounts for more than 90% of malignancies of the oral cavity with multifactorial and multistage etiopathogenesis.¹ In the past 30 years, despite advances in diagnostic techniques and treatment modalities, the incidence and prevalence of oral SCC have been on the increase, mainly in young adults.² The stage of SCC in the oral cavity at the time of diagnosis is the most important factor for its prognosis, which is determined using the TNM system. This system works by integrating the three basic clinical properties of tumor size, metastasis to lymph nodes and distant metastasis. In this context, the smaller the size of the initial tumor and the absence of any evidence of regional lymph node involvement favor a better prognosis, and the bigger the size of the initial tumor and involvement of regional or distant lymph node indicates a worse prognosis.^{2,3}

Previous studies on SCC have denoted relationships between several factors regarding the tumor

and the risk of its metastasis to lymph nodes along with the survival rate of the patients. The factors assessed including clinical and anatomical properties of the tumor, such as the location and extent of the tumor, the stage and the degree of histological differentiation of the initial tumor, the thickness of the tumor and its invasion to vessels and nerves.^{3,4} One of the factors that have been assessed in relation to the risk of metastasis to cervical lymph nodes that affects the prognosis of oral squamous cell carcinoma (OSCC) is inflammatory cells, especially eosinophils.⁴ The inflammatory infiltration in the tumor tissue matrix consists of primarily monocytic cells and low amounts of neutrophils and eosinophils.^{5,6} Eosinophils originate from the bone marrow and account for 1-3% of the leukocytes in the peripheral blood flow.⁷ These cells are characterized by specific round and ovoid shaped cells containing cytotoxic granules including major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophilic peroxidase (EPX) and eosinophil cationic protein (ECP). Those protein granules are found in the cell cytoplasm and their

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two-lobed nucleus. They are deeply stained with eosin and clearly visible under a light microscope.⁸ It should be pointed out that eosinophils have the potential to synthesize and secrete inflammatory mediators such as GM-CSF, IL-3, IL-5, TGF- β , TNF- α and TGF- α , which play a role in the lysis of cells including tumoral cells.^{9,10}

Tumor-associated tissue eosinophilia (TATE) is defined as the infiltration of inflammatory cells with eosinophilic predominance around or within the tumor. Such inflammatory infiltration has been found to be present in many areas, for instance, the pharynx, larynx, lungs, stomach and the oral cavity.^{9,11-13} A large number of studies have analyzed TATE status and its relationship with prognosis and the patients' survival rate.¹²⁻¹⁶ However, contradictory results have been reported on the relationship between eosinophilic infiltration and clinicopathological factors as well as the prognosis of head and neck SCC (HNSCC).¹⁴⁻¹⁹ Since the majority of studies have reported TATE as a favorable prognostic indicator, theoretically, it might be possible in the future to use immunotherapy with the assistance of the anti-tumoral feature of eosinophils to treat cancers.²⁰

Based on what has discussed above regarding the discrepancy between the results of previous studies, the present study was aimed to evaluate tissue eosinophilia in patients with HNSCC and its relationship with the histological differentiation of the tumor and other clinicopathological characteristics. Luna histochemical staining technique was used in this study to stain all the eosinophilic granules red, contrasting with the underlying blue background to promote better visibility under the microscope. This staining technique is specific for eosinophils and is commonly used to identify them in tissues.^{21,22}

MATERIALS AND METHODS

In the present cross-sectional study, 107 files of patients with HNSCC (including oral cavity, pharynx, larynx, and lips) were evaluated and were sorted further based on the following criteria:

1. The lesion was treated with surgical technique only
2. The lesion was a primarily found lesion with no report of recurrence
3. The patient has no history of radiotherapy and chemotherapy
4. The sample is provided with complete demographic data of the patient

Histological cross-sections were prepared from the selected samples, and the hematoxylin

and eosin (H&E) slides were categorized in terms of tumor differentiation grades based on Broder criteria.³ A nasal polyp sample was used as a positive control for evaluation of eosinophil staining. A total of 67 final slides were evaluated to determine eosinophil counts. The relevant paraffin blocks of the final samples were sectioned into 4 μ m cross-sections using a microtome (Leitz, Germany), selected within the approximate tumoral tissue site and prepared for histochemical staining. The cross-sections slides were transferred to alcohol to remove any wrinkles and then transferred to lukewarm water (45-48°C). Appropriate cross-sections were retrieved from the surface of the water with the use of lamellae coated with albumin glue. Cross-sections spread on lamellae were cautiously dehydrated and placed in an autoclave at 60°C for half an hour to remove paraffin so that the tissue would properly adhere to the lamellae. To remove the embedded paraffin completely, the samples were then immersed in xylol for 20 minutes. After the samples were deparaffinized, they are ready for Luna staining.

In the first step of Luna's staining method, the samples were dyed with a mixture of hematoxylin and Biebrich scarlet for 5 minutes. Subsequently, they were rinsed with 1% acid alcohol solution (hydrochloric acid), followed by rinsing in water. In the next stage, the samples were rinsed with lithium carbonate solution and rinsed in running water for 2 minutes afterward.¹² Finally, 95% and absolute alcohol were used for dehydration. In the final stage, the samples were covered with lamellae. In order to determine eosinophil counts in the connective tissue within the tumor, ten areas within the tumor were selected in a random manner under a light microscope (Olympus, Japan) and eosinophil counts were determined in each area at 400x magnification, and the sums of these counts were categorized into 4 groups.²³

0 eosinophils found: absence of eosinophils
 1-4 eosinophils found: poor infiltration
 5-19 eosinophils found: moderate infiltration
 ≥ 20 eosinophils found: severe infiltration

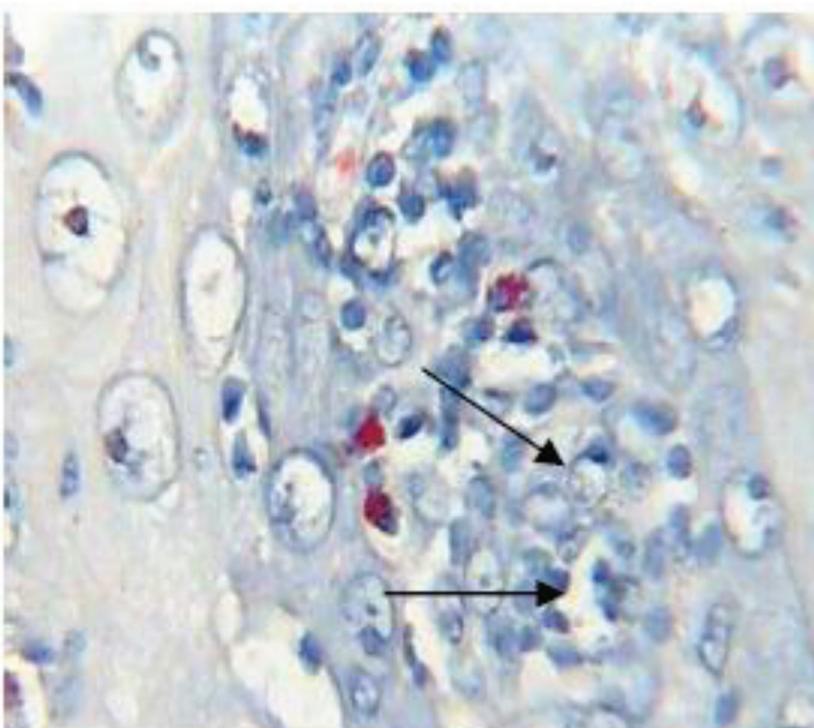
Statistical analyses were carried out with SPSS 19, using non-parametric tests, Mann-Whitney, chi-square and Kruskal-Wallis tests and Spearman's correlation coefficient. Statistical significance was set at $p < 0.05$.

RESULTS

Of all the 67 HNSCC samples gathered in the present study, 41 (61.2%) were male patient with a mean age of 56.6 \pm 13.1 years old, and 26 (38.8%) were female patient with a mean age of 62 \pm 14.7 years old.

Table 1 The frequencies of TATE in the head and neck SCC, in term of the relevant factors

Eosinophil count in 10 HPF		0	Poor	Moderate	Severe	Total	p-value
Histopathologic degree The degree of differentiation	good	0	0	4 (5.79)	21 (31.34)	25 (37.30)	0.04*
	Poor	1 (1.49)	1 (1.49)	5 (7.46)	23 (34.32)	30 (44.77)	
	Severe	0	2 (2.98)	5 (7.46)	5 (7.46)	12 (17.91)	
Age	30-44	0	1 (1.49)	2 (2.98)	6 (8.95)	9 (13.43)	0.28
	45-59	0	2 (2.98)	9 (13.43)	21 (31.34)	32 (47.76)	
	60-74	1 (1.49)	0	0	13 (19.40)	14 (20.89)	
	75-90	0	0	3 (4.47)	9 (13.43)	12 (17.91)	
Location	Tongue	0	0	3 (4.47)	14 (20.89)	17 (25.37)	0.45
	Mouth floor	0	0	0	4 (5.79)	4 (5.79)	
	Lips	0	0	2 (2.98)	10 (14.92)	12 (17.91)	
	Farenx	0	1 (1.49)	4 (5.79)	7 (10.44)	12 (17.91)	
	Lorenx	1 (1.49)	1 (1.49)	4 (5.79)	11 (16.41)	17 (25.37)	
	Glott	0	0	1 (1.49)	1 (1.49)	2 (2.98)	
	Other areas	0	1 (1.49)	0	2 (2.98)	3 (4.47)	
Gender	Male	1 (1.49)	3 (4.47)	9 (13.43)	28 (41.79)	41 (61.19)	0.07
	Female	0	0	5 (7.46)	21 (31.34)	26 (38.8)	

**Figure 1** Oral squamous cell carcinoma, eosinophilic (arrows), coloring Luna Magnify $\times 400$

The highest frequency was noted in the 45-59 years of age group with 32 (47.8%) cases. The oldest subject was 90 years old, and the youngest was 36 years old. The most frequent locations of the tumor

were on the tongue and the pharynx, and the least frequent location was on the larynx. All the samples were histologically graded in three groups: 37.3% samples were well differentiated ($n = 25$ cases); 44.8% samples were moderately differentiated ($n = 30$ cases); and 17.9% samples were poorly differentiated ($n = 12$ cases).

The mean count of eosinophils in each sample was 5.41 ± 4.74 (Figure 1). The total eosinophil counts in 10 high-power fields (HPF) were as follows: No infiltration (0): one case (1.2%), poor infiltration: 3 cases (3.6%), moderate infiltration: 14 cases (16.9%), severe infiltration: 49 cases (59%) as showed in Table 1. Kruskal-Wallis test showed a significant relationship between histological grades and eosinophil counts ($p = 0.04$).

DISCUSSION

The course of cancers, including OSCC, depends on several factors such as the host's immune response to the tumor. In this context, one of the most common cells in inflammatory infiltration against tumor cells is eosinophils. These cells secrete cytotoxic proteins such as MBP and ECP and are capable of activating the immune system through the release of some interleukins such as IL-2, IL-4, and IL-5, resulting in the reinforcement of the immune system and combat against the tumor; however, the release of IL-10 and TGF- β contributes to tumor

progression.^{12,24} The result of the present study showed a significant relationship between histological grading and TATE ($p = 0.04$), which means there was a decrease in TATE with higher histological grading and poorer differentiation of the tumor, confirming the anti-tumoral role of eosinophils.

On the other hand, some studies reported conflicting verdicts. For example, Leighton et al. evaluated TATE and its effect on nasopharyngeal carcinoma. In that study, TATE was categorized into several groups based on eosinophil counts: poor (<10), moderate (10-100) and severe (>100); along with result indicating no relationships between TATE and factors involved in the tumor prognosis.²⁵ In another study by Tadbir et al., the role of TATE was appraised as a factor to determine the prognosis in OSCC; the result showed no significant relationship between TATE and tumor differentiation.²⁶ In addition, in two separate studies, Goldsmith et al. (1987 and 1992) evaluated TATE and eosinophils in head and neck malignancies and deduced that eosinophils were associated with favorable prognosis. Despite that, the relationship between TATE and histological grading of the tumor was not evaluated in those studies.^{17,18} The discrepancies between the results of one study to another might be attributed to variation in the techniques used to determine eosinophil counts, the staining techniques used and the grading system used to specify the eosinophil counts.

In all of the studies discussed above, hematoxylin and eosin (H&E) staining technique was used despite there are other staining methods with higher sensitivity. However, other techniques including auto fluorescence or non-histochemical techniques have been used in some studies to identify sound or degranulated eosinophils in the tumor. The use of those other staining methods is due to several reasons, such as the presence of unusual and uncommon morphology of granulocytes particularly eosinophils, examining fibrotic tissue or severely infiltrated samples, and difficulty identifying the cells on the microscopic plates.²⁷ For example, in a study by Joshi et al., Congo red staining technique was used for identification of eosinophils, which was significantly more effective than H&E for identification of these cells.²⁴ The study by Dorta et al. also showed no significant distinction in the mean counts of eosinophils in each millimeter-square surface area between H&E and immunohistochemical (IHC) staining techniques in OSCC. Nevertheless, it was reported that when a monoclonal antibody is used, CD125 of this antibody reacts with only one epitope on the molecule, resulting in the reaction of antibodies with a limited number of antigens; therefore, the number

of eosinophils identified by IHC is less than that by H&E staining.²⁷ Other than that, a study of the effect of angiogenesis and immune cells on the SCC of the tongue with the use of carbol chromotrope by Alkhabuli showed no relationship between eosinophil concentration and histological grading of the tumor.¹² Into the bargain, Luna histochemical staining was used in the present study owing to its lower cost, relatively easily accessible compared to other techniques, and its ability to stain eosinophils selectively hence increasing the accuracy of the speed of the evaluation.²¹ As discussed previously, no significant relationship was demonstrated between TATE and variables such as gender and tumor location, consistent with the results of the study by Oliveira et al., in which no significant relationship was found between TATE and gender, ethnicity, tumor location and tumor stage. In that study, tumors with higher and more severe TATE were significantly associated with a history of alcohol use and smoking.²⁸

Many of the studies mentioned above have reported inconsistent results in HNSCC, and there is still no definitive conclusion available relating to the presence of eosinophils in the HNSCC. The differences in the results might be ascribed to the reasons conferred above, including the tumor location and other factors. A new consideration in the present study was the use of Luna staining technique. Unfortunately, due to the lack of adequate access to patient data and sufficient information in the patients' files, it was not possible to evaluate the rates of recurrences, survival, and metastasis.

CONCLUSION AND RECOMMENDATIONS

It appears that tissue eosinophils decrease significantly with a decrease in the differentiation of tumor cells in the head and neck SCCs.

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