Original Article

Tumor-Associated Tissue Eosinophilia in Oral Squamous Cell Carcinoma: Implications for Histopathologic Grading

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ABSTRACT

Tumor-associated tissue eosinophilia (TATE) has been associated with various tumors, including oral squamous cell carcinoma. However, the precise role of TATE in these contexts remains to be fully elucidated. The present study was undertaken to investigate the presence of tumor-associated tissue eosinophilia in oral squamous cell carcinomas (OSCCs) and its correlation with histopathologic grading. A total of 70 OSCC tissue samples were collected between 2016 and 2020 for examination. The samples comprised 60 previously diagnosed cases of OSCC, classified as well-differentiated squamous cell carcinoma (WDSCC), moderately differentiated squamous cell carcinoma (MDSCC), and poorly differentiated squamous cell carcinoma (PDSCC). Two observers independently assessed TATE using Sirius red stain, with the average eosinophil count evaluated in 10 fields under the ×40 objective lens. Statistical analysis involved the Student t-test, One-way ANOVA, and chi-square test. The study encompassed 70 OSCC samples and corresponding healthy tissue, with OSCC predominantly found in the tongue, representing 61.4% of cases. The total eosinophil count per high-power field (HPF10) was significantly higher in oral squamous cell carcinoma (OSCC) compared to healthy tissue. Furthermore, the mean TATE score was found to be considerably elevated in OSCC tissue. However, a one-way analysis of variance (ANOVA) revealed a statistically non-significant association between different grades of OSCC and eosinophil counts. However, the chi-square test did not indicate a significant association between eosinophil count and gender or age group. The present study underscores the heightened eosinophil count observed in cancerous tissues compared to healthy tissues. However, the variability in eosinophil counts across distinct OSCC grades remains ambiguous. Further investigation is recommended to delve into the infiltration of eosinophils in solid tumors and their potential role in predicting malignancies, particularly in OSCC.

Keywords: Oral Squamous Cell Carcinoma, Sirius Red, Eosinophils, Stains, Tumor-Associated Tissue Eosinophilia.

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a type of cancer that affects the mouth, including the tongue, lips, gums, and floor [1-4]. This condition has prompted significant health concerns on a global scale, primarily due to its impact on patients' quality of life. Despite strides in diagnostic methods and therapeutic interventions, OSCC persists as a formidable health challenge, exhibiting a five-year survival rate of only 50% [5, 6]. In recent years, there has been an escalating interest in elucidating the role of the immune system in the progression of oral squamous cell carcinoma (OSCC). Research findings have indicated that eosinophils within the tumor microenvironment play a role in tumor growth and invasion [9, 10]. While eosinophils are traditionally associated with reactions and parasitic infections, they have also been implicated in the development of various cancers, including OSCC [11]. The presence of eosinophils within the tumor microenvironment (TAME) has been explored as a potential marker for various types of cancer. In the context of OSCC, TATE has been associated with survival outcomes, suggesting a potential role for eosinophils in the progression of this disease [12, 13]. Conventionally, the evaluation of TATE has relied on the analysis of tissue samples, a process that can be subjective and may not accurately reflect the presence or extent of TATE. However, recent advancements in special staining techniques, such as immunohistochemical staining and Giemsa staining, have emerged to enhance the detection and quantification of eosinophils in tissue specimens. These techniques utilize antibodies to identify eosinophils and stain the cytoplasmic granules of eosinophils, respectively [14]. Despite the fact that several studies have evaluated the presence of TATE in OSCC, further research is necessary to determine how TATE varies across different grades of OSCC. The classification of OSCC is based on the degree of differentiation of the tumor cells, with higher stages indicating more aggressive tumors. A comprehensive understanding of TATE's variation across diverse OSCC sites could offer crucial insights into the role of eosinophils in cancer progression and the potential utilization of TATE as a prognostic marker [9, 15, 16]. The present study aims to evaluate the presence and extent of TATE in different grades of OSCC using special staining techniques. Specifically, immunohistochemical and Giemsa staining techniques will be employed to identify and quantify eosinophils in tissue specimens from patients with varying grades of OSCC. The findings of this study could offer significant insights into the role of eosinophils in OSCC and their potential as a prognostic marker for this disease.

2. Materials and Methods

2.1. Study design

This laboratory study analyzed 70 tissue samples, including 60 cases of oral squamous cell carcinoma (OSCC) and 10 samples of normal tissue. The tissue blocks were obtained from the archives of the Cancer Institute at Imam Khomeini Hospital, Tehran University of Medical Science, Iran, between the beginning of 2016 and March 2020. Following histopathological diagnosis, the OSCC tissue samples were stratified into three groups: Group 1 consisted of 20 cases of well-differentiated OSCC (WDSCC); Group 2 had 20 cases of moderately differentiated OSCC (MDSCC); and Group 3 included 20 cases of poorly differentiated OSCC (PDSCC).

2.1.1. Inclusion Criteria

The present study included individuals diagnosed with different grades of OSCC through histopathological examination. The clinical data of the sample, including age, sex, habits, and lesion site, were obtained from the records.

2.1.2. Exclusion Criteria

Patients with other concurrent primary tumors or metastatic tumors of the oral cavity or jaw bones will be excluded from the study. Individuals with a documented history of chemotherapy and radiotherapy will also be excluded from the study. The histological sections were classified according to Broder's criteria for differentiation. To prepare for histochemical staining, 2-micron sections were obtained from selected paraffin blocks using a Japanese Sakura microtome machine. A sharp cutting blade was utilized to obtain suitable slides, and blocks exhibiting a smooth surface were documented. The obtained sections were then transferred to an alcohol-water solution (comprised of one part 96-degree alcohol and two parts water) using tweezers, with the objective of removing any tissue wrinkles. Subsequently, the cut strips were transferred to lukewarm water (45 to 48 degrees Celsius), and a slide composed of the relevant sections that had been dipped in albumen glue was removed from the water. The slices were then subjected to a process of dewatering, a procedure that involved the removal of excess moisture. Following this step, the slices were placed in an autoclave, a specialized apparatus used for high-temperature sterilization. The autoclave was set to 60 degrees Celsius, which is equivalent to the melting temperature of paraffin. The slices were maintained within the autoclave for a duration of half an hour, a time sufficient to ensure the complete dissolution of the paraffin and the optimal adhesion of the tissue to the slide. The slides were then placed in a Gezilel container for a period of twenty minutes, during which the tissue paraffin was removed. For Sirius Red staining, deparaffinized slides were first immersed in alcohol. Subsequently, the slides were immersed in hematoxylin for a period of two minutes, followed by a thorough rinse. Subsequently, the slides were immersed in a 1% alcohol acid solution (hydrochloric acid) for a duration of 5 seconds and then thoroughly rinsed with water. The following step involved placing the slides in 100% alcohol, in Sirius Red alkaline dye (PH=8-9), for a period of two hours. Following this step, the slides were thoroughly washed with running water for a duration of three minutes. Thereafter, they underwent a process of dehydration using alcohol, followed by clarification with xylyl. To protect the tissue, coverslips were affixed onto the slides.

2.2. Eosinophil Counting Method in Tumor Connective Tissue

The quantification of eosinophil cells within the connective tissue of the tumor was conducted using an Olympus C21 microscope (Japan). A total of ten random areas were selected within the tumor, and the number of eosinophil cells in each area was counted at a magnification of 400. The mean number of tissue eosinophils (TATE) in these areas was subsequently calculated and categorized into three distinct classes as outlined in the literature [17]:

-Grade 0: (0-2)

-Grade 1: (3-10)

- Grade 2: (11-20)

2.3. statistical Analyses

The statistical software program SPSS version 21.0 was utilized to analyze the data with the aid of Student's t-test, one-way ANOVA, and chi-square. A P-value of less than 0.05 was considered statistically significant. Continuous data were described as mean±standard deviation (SD), while categorical data were expressed as frequency and percentage.

2.4. Ethical Consideration

The study was approved by the Qazvin University of Medical Science's research ethics committee (IR.QUMS.REC.1399.060). The data collected were exclusively utilized for the purposes of this study, and the confidentiality of the participants was maintained at all times.

3. Results

Of the 70 samples of oral squamous cell carcinoma (OSCC) and healthy tissue included in the study, 48 (68.6%) were male with a mean age of 56.9±5.16 years, and 22 (31.4%) were female with a mean age of 63.69±3.6 years. The OSCC cases were categorized based on OSCC incidence and grading, as shown in Table 1. The age of the participants ranged from 31 to 91 years, with a mean age of 62.65 ± 6.15 years reported for all participants. The tongue was the most commonly affected site, accounting for 43 cases (61.4%), while the least affected area was the hard palate, with only one patient (1.4%) reported (Table 1). The total mean count of eosinophils per HPF10 is as follows: zero (2-0): 29 cases (41.4%), 1+ (10-3): 36 cases (51.4%), and 2+ (20-11): 5 cases (7.1%) (Figure 1). Additionally, the mean TATE was estimated at 31.1±0.52 in healthy tissue but was significantly higher at 71.3±27.5 in OSCC tissue. Table 2 presents a frequency table that illustrates the quantity of tissue eosinophils in HPF10 and the tissue type. The Student t-test revealed a significant increase in the number of tissue eosinophils in OSCC compared to healthy tissue (P=0.026). However, the one-way ANOVA test (P=0.048) suggests that there is no significant association between different OSCC grades and the number of eosinophils. However, due to the limited sample size in each grade, it is challenging to ascertain a precise trend regarding the decrease or increase in eosinophils. The association between gender and the total number of eosinophils was evaluated using the chi-square statistical test, which showed no significant association (P=0.8). A similar outcome was observed when the chi-square test was employed to assess the relationship between age group frequency and the total number of eosinophils; this analysis yielded a P-value of 0.15, indicating that the observed association was not statistically significant. The final objective of the study was to investigate the association between the location of the tumor and eosinophils. The analysis revealed that a greater proportion of the samples were associated with the tongue, with a comparatively smaller proportion originating from other regions, such as the hard palate and mandibular bone. Consequently, the statistical significance of the relationship between eosinophil levels and tumor location could not be determined.

4. Discussion

Oral squamous cell carcinoma (OSCC) has emerged as a significant global health concern, impacting thousands of individuals annually. Consequently, the identification of factors associated with its prevention is of paramount importance. The tumor microenvironment, including stromal cells, has emerged as a pivotal area of research in cancer studies. The tumor stroma reflects essential features such as angiogenesis, inflammation, and invasion, with infiltration of inflammatory cells into the tumor stroma being observed in most tumors [3, 5, 9, 15, 16, 18]. Among the immune response and inflammatory cells, TAGE has been the focus of studies in various cancers, including the nasopharynx, larynx, lung, digestive system, oral cavity, and esophagus. However, the precise function of TATE in tumor progression remains to be fully elucidated by previous studies [19]. Eosinophils are cells with dual functions that can play a role in the stimulation and destruction of tumors. In the presence of eotaxin-1 production by a tumor cell, eosinophils are attracted to the tumor site. The release of granule proteins, including neurotoxins, peroxidases, and cationic proteins, along with an increase in the permeability of tumor cells to these cytokines, leads to the destruction of the tumor [9]. It has been suggested that TATE can play a role in tissue remodeling, particularly in damaged muscle fibers due to the invasion of malignant cells [20]. Conversely, TATE has been observed to stimulate tumor angiogenesis by producing angiogenic factors. Furthermore, the presence of precursor enzymes such as MMP-9 and their inhibitors, including TIMP-1 and TIMP-2, suggests a regulatory role for these cells in the formation of the extracellular matrix [21]. Given the established role of eosinophils in angiogenesis and extracellular matrix, these cells may also contribute to tumor invasion and progression. The present study investigated the presence of eosinophils in healthy tissue and oral squamous cell carcinoma (OSCC), as well as the association between the numbers of eosinophils in different tumor histology degrees. The study examined a total of 60 samples from patients with OSCC and 10

Table 1. Frequency of sex, tissue type, histopathology grade, and site in the samples examined

		Number	Percentage
gender	Man	48	68.6
	Female	22	31.4
tissue type	Healthy tissue	10	14.3
	Well-differentiated SCC	20	28.6
	Moderately differentiated SCC	20	28.6
	Poorly differentiated SCC	20	28.6
TATE	zero degree	29	41.4
	Grade 1+	36	51.4
	Grade 2+	5	7.1
Site	lingual	43	61.4
	the floor of the mouth	3	4.3
	lower lip	12	17.1
	Mandibular bone	2	2.9
	buccal mucosa	9	12.9
	hard palate	1	1.4

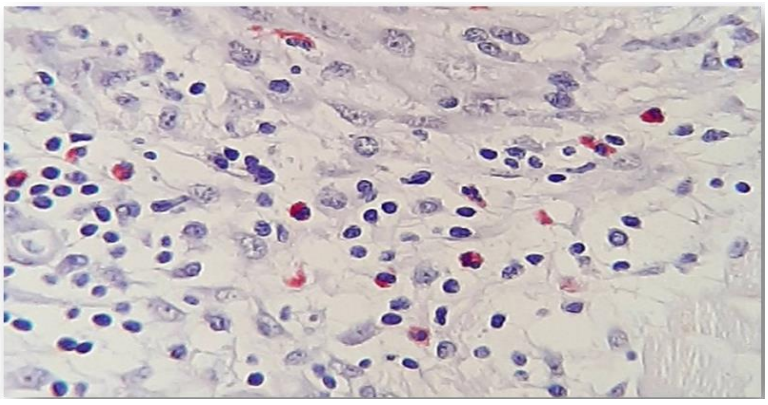

Figure 1. Oral squamous cell carcinoma, eosinophil (arrow), Sirius Red staining, magnification 400x

Table 2: The relationship of TATE with tissue type, histopathological grade, lesion site, and gender.

TATE		**0**	**+1**	**+2**	**overall**	**P.value**
tissue type	**normal**	8 (% ١١/٤)	٢ (%٢/٩)	٠ (%٠/٠)	١٠ (%١٤/٣)	* ٠٢٦.٠
	Degree of distinction Squamous cell carcinoma	٢١ (%٣٠)	٣٤ (%٤٨/٦)	٥ (%٧/١)	٦٠ (%٨٥/٧)	
Degree of distinction Squamous cell carcinoma	**well differentiated**	٤ (%٥/٧)	١٤ (%٢٠)	٢ (%٢/٩)	٢٠ (%٢٨/٦)	0.048
	moderately differentiated	١١ (%١٥/٧)	٨ (%١١/٤)	١ (%١/٥)	٢٠ (%٢٨/٦)	
	poorly differentiated and undifferentiated	٦ (%٢٠/٧)	١٢ (%١٧/١)	٢ (%٢/٩)	٢٠ (%٢٨/٦)	
gender	**female**	٢٠ (%٢٨/٦)	٢٤ (%٣٤/٣)	٤ (%٨/٣)	٤٨ (%٦٨/٦)	0.8
	male	٩ (%١٢/٩)	١٢ (%١٧/١)	١ (%٤/٥)	٢٢ (%٣١/٤)	

samples from healthy tissue. The demographic composition of the collected samples revealed a male-to-female ratio of 2.18:1, with 68.6% of the samples originating from male subjects and 31.4% from female subjects. According to epidemiological studies, the incidence of OSCC is higher in men than in women, and the overall male-to-female incidence ratio is now 2 to 1. In the present study, the majority of the samples (61.4%) were from the tongue. This finding aligns with the epidemiological studies that have identified the tongue as the predominant site for oral malignancies. In other studies, TATE and various factors determining the prognosis of SCC have been investigated, yielding different results. Notably, the present study observed a significantly higher number of tissue eosinophils in OSCC compared to healthy tissue, as reported in previous studies [24, 26]. Concurrently, other researchers have obtained analogous results in their studies, which compared the number of tissue eosinophils in SCC and healthy tissue. A number of studies have identified a statistically significant discrepancy in the increase of tissue eosinophils between cancerous and healthy tissue [24, 26]. Additionally, Saravani et al. observed a higher average number of eosinophils in OSCC compared to precancerous oral lesions [27]. Furthermore, Razavi et al. found that the number of tissue eosinophils in OSCC is higher than in dysplastic tissue, and in dysplastic tissue, it is higher than in healthy tissue [28]. In the present study, the overall mean TATE in OSCC samples was 5.27, while in healthy tissue, it was 2.05. The eosinophil count was found to be significantly higher in cancerous tissue compared to healthy tissue. In a separate study by Saravani, the mean TATE was reported to be 90 in OSCC and 11.08 in one type of precancerous lesion [27]. In a separate study by Shani et al., the mean TATE in OSCC and healthy tissue was reported to be 3 and 2, respectively [24]. Razavi et al. reported 0.25, 12, and 74 in healthy, dysplastic, and SCC tissues, respectively [28]. Consequently, these studies have led to the conclusion that eosinophils can be regarded as an adverse prognostic factor and a potential catalyst for the transformation of precancerous lesions into malignant ones. A critical factor in predicting SCC is the degree of tumor histopathological differentiation, which was classified into three categories based on Border's criteria in this study [29]. Concerning the relationship between TATE and SCC differentiation grade, the findings of this study indicated an absence of a statistically significant correlation between these two factors. However, due to the limited sample size in each group, the precise trend of increase or decrease in the number of eosinophils could not be ascertained. Consequently, the outcomes of this study can be consistent with those of other studies [19, 26, 27, 30] because no significant difference was observed in the presence of eosinophils in different degrees of SCC histology in these studies. Jain et al. [30] further underscores the significance of routine TATE examination in patients with SCC, though it remains unable to elucidate the underlying mechanism of TATE's inhibitory effect on tumors. This observation aligns with the findings of the present study. Conversely, contradictory results have been reported in other studies. Majumdar et al. [18] and Shani et al. [24] reported on the role of anti-tumoral tissue eosinophils, and according to their results, the number of tissue eosinophils was higher in SCC samples with good differentiation. Vaibhav et al. [31] also discussed the role of eosinophil stimulants in the tumor mechanism. Their findings indicated that the number of tissue eosinophils would also be higher when the tumor was more prominent and had a higher grade and stage. In other words, the lower the differentiation of tumor cells, the higher the number of tissue eosinophils. In a similar vein, Razavi et al. [28] noted that TATE levels escalate in response to the progression of dysplastic tissue from mild to severe stages or the progression of squamous cell carcinoma (SCC) towards higher degrees of differentiation. The discrepancies observed in the results of recent studies when compared to our findings may be attributable to various factors, including differences in counting and categorizing eosinophils, the sites of examination, the staining methods employed, variations in sample sizes, and potentially genetic and racial disparities among the study participants. Vaibhav et al. [31] and Majumdar et al. [18] utilized cabal-chromo trope staining in their respective studies, and Vaibhav et al. [31] also employed unequal numbers of samples in each grade (good differentiation: 30, moderate differentiation: 20, poor differentiation: 10). In contrast, the staining method employed in our study was Sirius red. The number of samples in each group was equal to 20. Additionally, Saravani [27] employed a distinct method, namely congo-red, utilizing a mere eight samples in each group. In a subsequent study, Razavi et al. [28] employed three staining methods: Sirius red, H&E, and one immunohistochemical staining. As previously stated, the Aladini classification system was utilized to categorize the counted eosinophils in our study. The mean number of TATE was then classified into four categories: zero: (2-0), 1+: (10-3), 2+: (20-11), 3+: (30-21), and 4+: (30 ≤). In contrast, other researchers, such as Vaibhav et al. [31], classified the number of tissue eosinophils into three categories: mild (less than 50), moderate (50-120), and intense (more than 120). In contrast, Razavi et al. [28] employed the mean number of TATE for comparison without categorizing it into distinct degrees. Sirius red staining, a technique that has been utilized in numerous studies, represents a novel component of our research. For instance, only Saravani et al. [27] employed this method in their research. Sirius-red staining is a non-repetitive staining method, and studies can continue to use it. Some researchers have employed congo-red staining in isolation or in conjunction with alternative methods. In this study, we employed photochromic staining with Sirius red, a relatively inexpensive and readily available method that specifically identifies eosinophil cells, with the objective of enhancing the efficiency and precision of slide reading. As previously mentioned, the advantage of using this staining is that even if eosinophils do not have their normal and

specific morphology, they are better identified with this method than other staining techniques such as H&E and Congo red [27]. The stained lamellae exhibit a distinctive blue background, allowing for the clear identification of eosinophils with their characteristic red cytoplasm and prominent blue nuclei, which are typically binuclear. As demonstrated in the aforementioned studies, divergent outcomes have been documented in the analysis of SCCs within the head and neck region. A definitive conclusion regarding the presence of eosinophils in SCCs of the head and neck remains elusive. The observed discrepancy among studies can be attributed to potential variations in methodology, including the technique for enumerating eosinophils, as well as other confounding factors.Notwithstanding, there is ample evidence supporting the presence of eosinophils as inflammatory cells associated with various cancers, particularly epithelial types such as OSCC. Nevertheless, further research is necessary to ascertain the precise role of eosinophils in tumor growth, patient survival, and their potential impact on malignancy progression. In this study, we sought to elucidate the disparities in the number of tissue eosinophils between healthy and cancerous tissues, as well as the variations in eosinophil count among different grades of squamous cell carcinoma (SCC). Our findings revealed that eosinophil levels were significantly elevated in cancerous tissues compared to healthy tissues. However, the precise pattern of change in eosinophil levels across different histological grades of cancer remains to be fully elucidated. However, considering the findings from other studies, it is crucial for future research to continue investigating eosinophil infiltration in solid tumors. This will facilitate the development of a comprehensive understanding of their role in these contexts. In the contemporary context, the advancement of an invasive malignancy is influenced not only by genetic alterations in tumor cells but also by changes in the host stroma, endothelial cells, and immune or inflammatory cells. Given the synergistic performance of eosinophils with macrophages and lymphocytes, it is recommended that future studies investigate the simultaneous presence of these cells. Furthermore, it is recommended to evaluate the presence of these cells along with angiogenesis in tumors and their effect on this process, considering the role of eosinophils in the phenomenon of angiogenesis and the effectiveness of the angiogenesis process in predicting malignancies such as OSCC.

Highlights

Oral squamous cell carcinoma (OSCC) poses a considerable health threat, with a low survival rate.
Eosinophils in the tumor microenvironment (TATE) have been linked to survival rates in OSCC.
Special staining techniques have improved the detection and quantification of eosinophils in tissue specimens.
This study aims to evaluate the presence and extent of TATE in different grades of OSCC using special staining techniques.

Importance of the Study

Understanding the role of eosinophils in OSCC and their potential as a prognostic marker.
Providing crucial insights into the association between TATE and cancer progression.
Evaluating the variation of TATE across different grades of OSCC.

Limitations of the Study

The analysis of tissue samples for TATE can be subjective and may not represent the true extent of TATE.
The number of samples in each grade of OSCC was limited, requiring further statistical analysis.
The association between eosinophils and tumor site could not be analyzed due to insufficient data.

Implication for Practice Statement

The results of this study may contribute to the development of prognostic markers for OSCC.
Understanding the role of eosinophils in cancer progression can guide treatment strategies.
Special staining techniques can be utilized in clinical practice to assess TATE in OSCC patients.

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Authors' Contribution

Conceptualization: M.H, A.T, A.M, and F.A; Data curation: M.H, A.T, A.M, and F.A; Formal analysis: Z.Y; Methodology: M.H, A.T, A.M, F.A, and Z.Y; Writing - original draft: M.H, A.T, A.M, F.A, and Z.Y; Writing - review & editing: A.M, and F.A.; All authors read and approved the final version of the manuscript.

Ethics

The study was reviewed and endorsed by the Research Ethics Committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1399.060).

Conflict of Interest

The authors declare that they have no known competing financial interests or personal associations that could have appeared to influence the work reported in this paper.

Supplementary Material

Supplemental materials may be obtained upon request to the corresponding author**.**

Data Availability

The data presented in this study are available upon request from the corresponding author.

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