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QUANTITATIVE ASSESSMENT OF TUMOR ASSOCIATED TISSUE EOSINOPHILIA IN ORAL SQUAMOUS CELL CARCINOMA (CLINICOPATHOLOGICAL STUDY)

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ABSTRACT

INTRODUCTION: Oral Squamous cell carcinoma (OSCC) is the 11th most common cancer worldwide. Tumor stroma consists of various inflammatory cells resulting from the host-response to tumor cell. Increased tissue eosinophil levels have been reported in various malignancies including OSCC. Tissue eosinophilia is a regular finding in allergic and parasitic disorders, but their role still needs to be evaluated in OSCCs. Specific stains, as Congo red is a useful diagnostic means for detecting eosinophils owing to their distinctive quality to combine with eosinophils.

OBJECTIVES: This study aimed to evaluate and compare eosinophilic infiltration in both metastatic and non-metastatic oral squamous cell carcinoma using Congo red stain and their correlation to the tumor grade.

MATERIAL AND METHODS: Fifty histopathologically proven OSCCs cases were included in this study and were allocated into 2 groups where the first group included 25 cases of metastatic OSCC and the remaining 25 cases are non-metastatic OSCC. Congo red was utilized as a distinct stain for eosinophils. Each specimen slide was inspected under high power in 10 successive microscopic fields to count eosinophils.

RESULTS: the eosinophilic count was found to be higher in non-metastatic tumors cases when compared to metastatic tumors cases.

CONCLUSION: eosinophilic count is a strong indicator for the stage of the tumor and its differentiation.

KEY WORDS: Carcinoma, Congo Red, Eosinophils, Eosinophilia, Mouth Neoplasms, Squamous Cell.

RUNNING TITLE: Quantitative assessment of tumor associated tissue eosinophilia in oral squamous cell carcinoma.

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INTRODUCTION

Oral Squamous cell carcinoma (OSCC) is the 11th most common cancer worldwide. There is a broad geographical disparity in the incidence of oral cancer, with approximately 66% of the involved patients being in developing countries, and very high mortality and morbidity rates.(1)

An international outlook on oral cancer is managed by the International Association of Cancer Registries (IARC), a section of the World Health Organization (WHO) through the GLOBOCAN project. The newest GLOBOCAN 2012 database was the source of data (2). A noteworthy discrepancy in the allocation of oral cancer across many WHO districts have been described (2).

In the Middle East and North Africa (MENA) area, recently diagnosed oropharyngeal cancer (OPC) and oral cavity cancer (OCC) cases were assessed in 2012 to be around 9000, which is almost 1.5% of all malignancies; yet the linked fatalities were about 3500 (1% of all malignancies). In the MENA area, the rank of OPC-OCCs is amid the top 20 cancers. New male cases were around 5000 whereas females were around 3800. The male to female ratio of mortality was 1.36:1 whereas incidence rate was 1.38:1in the entire MENA area (3, 4).

The chief risk factors in addition to genetic predisposition are heavy smoking, consumption of alcohol, infection by oncogenic viruses as human papilloma virus (HPV). Moreover, other risk factors may also have a part in carcinogenesis in the area, for instance; work-related, nutritional, and environmental factors. Alcohol consumption role is shadowed by religion and societal barriers; that's why Information on alcohol consumption and oral cancer is absent in the MENA area. (5)
OSCC is characterized by high local invasion rate and cervical metastasis (6). Lymph node association is detectable in 50% of patients at diagnosis. After five years, survival rate is less than 40% for this group, compared to a 90% survival rate for patients with no metastasis; in other words, the presence of nodal metastasis reduces the survival rate by almost 50% (7, 8).
In 1846, Wharton Jones first described eosinophils as "coarse granular cells", while in 1880 Paul Ehrlich referred to them as "eosinophils".(9) Eosinophils are characterized by existence of ample cytoplasm with coarse reflective granules(10) and are characterized by their tinctorial qualities displaying bright red staining with acid aniline dyes.(11) They are pleiotropic-multifunctional leucocytes and have a crucial part in disease and health, as they are engaged in commencement and spread of various inflammatory responses counting bacterial and viral infections, allergic diseases, parasitic helminth, and tissue injury in addition to regulators of adaptive and innate immunity.(12) Many cancers, together with OSCC, have also shown extensive tissue eosinophilia.(13)
Tumor-associated tissue eosinophilia (TATE) is described as "eosinophilic stromal infiltration of a tumor not associated with tumor necrosis or ulceration." In 1896, Przewoski was the first to describe it in cervix carcinoma.(14) It is distinguished by the existence of eosinophils being a constituent of intra-tumoral and peritumoral inflammatory infiltrate.(15, 16) In malignancies, TATE is linked with several locations such as nasopharynx,(14, 17) larynx,(11, 18) esophagus,(19) colon,(20, 21) cervix,(22) external genitalia,(23) skin,(24) gastrointestinal tract,(25) and oral cavity(15, 16, 23).
Theories have been present of eosinophils having direct tumoricidal action linked with discharge of cytotoxic proteins, also acting indirectly by increasing tumor cells permeability enabling infiltration of tumor-killing cytokines. Moreover, tumor angiogenesis may be boosted through the formation of several angiogenic factors. They also have preformed matrix metalloproteinases (MMP) such as MMP-9, along with their inhibitors TIMP-1 and TIMP-2 demonstrating that they can also regulate the formation of extracellular matrix.(26)
Tissue eosinophilia is a regular finding in allergic and parasitic disorders, but their role still needs to be evaluated in OSCCs.(19) Eosinophils are present in large numbers in some OSCC of the oral cavity, lower colon and cervix.(27) Eosinophils release chemical substances under diverse stimuli, such as interleukins, chemokines (RANTES, endotoxin1), eosinophil chemoattractant protein, major basic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. These substances may induce inflammation, cell death and impact tumor microenvironment. (28)
The predictive significance of eosinophils in oral carcinoma continues to be ambiguous; also TATE in the head and neck area presents debatable outcomes when used as a surrogate marker in expectation of recurrence and survival in OSCC.(29-31)

MATERIAL

STUDY SAMPLE

The current study included 50 specimens from OSCC cases diagnosed at oral pathology department, faculty of dentistry, Alexandria University, which was surgically excised with concomitant neck dissections at Oral and Cranio-maxillofacial and plastic surgery department, faculty of dentistry, Alexandria university.
A sample size of 25 samples per group (number of groups = 2) (total sample size = 50samples) is the enough required sample as statistically significant with 80% power and at a significance level of 95% (accepted error = 0.05). Sample size per group does not need to be increased to control for attrition bias.(32) The sample size was calculated using GPower version 3.1.9.2. (33)
Inter- and intra-examiner reliability when tracing the remaining wound area was calculated by intraclass correlation coefficient (ICC).(34)

METHODS

SAMPLES GROUPING

Samples collected were grouped according to lymph node involvement after studying the hematoxylin and eosin stained sections of all cases into 2 groups:

- **Group I:** non-metastatic OSCC (n=25).
- **Group II:** metastatic OSCC (n=25).

CONGO RED STAINING PROCEDURES

Initially, slides were deparaffinized, hydrated through graded alcohols to water, then left in 1% Congo red solution for 8 minutes ensued by rinsing in water. Next differentiation was done in 2.5% Potassium hydroxide (KOH) solution by dipping once. Slides was counterstained with hematoxylin for 8 minutes then rinsed under running tap water. Differentiation was done in 1% acid alcohol by dipping once. Finally, the slides were dehydrated through alcohol and cleared in xylene, then they were mounted with distyrene, a plasticizer, and xylene (DPX), which is a mixture that replaces xylene-balsam and used as a synthetic resin mounting media.

COUNTING OF EOSINOPHILS

Each specimen was inspected under high power (40x) microscopic field for counting of eosinophils. Diameter of high power field microscope used was 0.5 mm. In case of OSCC, invasive front region was chosen for eosinophils estimation. Ten consecutive high-power fields (hpf) were used to count the eosinophils, and the result was recorded as eosinophils/10hpf.(20) Areas of tumor necrosis and degenerated muscle tissue areas was excluded.

STATISTICAL ANALYSIS

Histologic observations were documented, and the results were tabulated and statistically analyzed.

RESULTS

CLINICAL DATA

The clinical data of this study is presented in Table 1. For the non-metastatic group, there were 11 females and 14 males with an average age of 55.6 ±7.57, while the metastatic group comprised 10 males and 15 females with an average age of 53.0 ±10.89. No significant difference has been found between the 2 groups as for age and gender.

The non-metastatic group comprised of 15 cases of tongue ulcer, 4 cases of buccal mucosa, 2 cases in the lower lip, and 4 cases in the floor of the mouth while the metastatic group comprised of 10 cases with tongue ulcer, 8 cases of buccal mucosa, 4 cases in the palate, and 3 cases in the retromolar area.

Table 1: Comparison between the two studied groups according to demographic data.

	Non metastatic (n = 25)		Metastatic (n = 25)		Test of Sig.	p
	No.	%	No.	%		
Gender						
Male	14	56.0	10	40.0	χ^2=1.282	0.258
Female	11	44.0	15	60.0		
Age						
Min. – Max.	42.0 –66.0		32.0 –74.0			
Mean ± SD.	55.6 ±7.57		53.0 ±10.89		t=0.980	0.332
Median (IQR)	58.0 (49.5 –61.5)		53.0 (44.0 –60.5)			

χ2: Chi square test t: Student t-test
p: p value for comparing between the studied groups

The non-metastatic group comprised of 13 well differentiated cases, 8 moderately differentiated cases and 4 poorly differentiated cases while the metastatic group comprised of 8 well differentiated cases, 8 moderately differentiated cases and 9 poorly differentiated cases.

As for the eosinophilic count (Figure 1), in the non-metastatic group, the average eosinophilic count was 8.38 ±1.82. there was a significant decrease in the eosinophilic count for the metastatic group where it was 4.3 ±1.43. When correlating the eosinophilic count to the gender (Table 2), there was no significant difference detected. However, in the metastatic group, the female eosinophilic count was significantly higher than that of the males.

Regarding the relation between differentiation and eosinophilic count (Table 3), the eosinophilic count significantly increased with the decrease in differentiation. However, the opposite was observed in the metastatic group where the eosinophilic count decreased significantly with the decrease in differentiation.

Finally, Table 4 presents the correlation between eosinophilia and age. There was no positive correlation between the eosinophilic count and age of the patients.

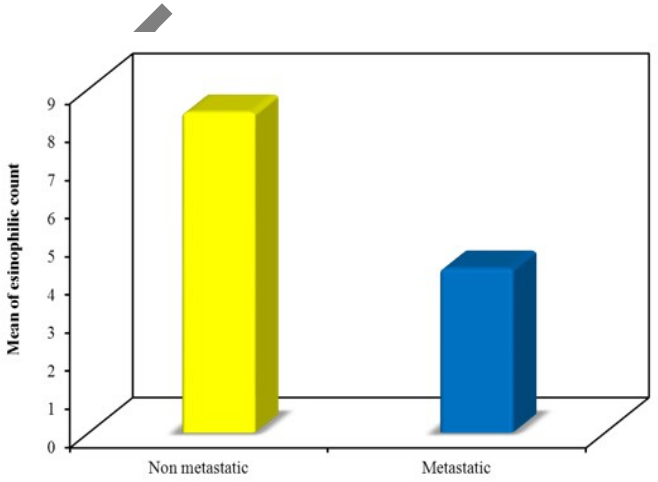

Figure 1: Comparison between the two studied groups according to eosinophilic count

Table 2: Relation between gender and eosinophilic count

Eosinophilic count	Gender		U	p
	Male	Female		
Non metastatic	(n = 14)	(n = 11)		
Min. – Max.	6.0 –13.0	6.80 –10.0		
Mean ± SD.	8.44 ±2.23	8.32 ±1.20	68.50	0.641
Median	7.60	8.10		
Metastatic	(n = 10)	(n = 15)		
Min. – Max.	2.5 –4.6	2.6 –7.0		
Mean ± SD.	3.21 ±0.70	5.02 ±1.34	21.0	0.003*
Median	2.95	5.40		

U: Mann Whitney test
p: p value for association between different categories
*: Statistically significant at $p \leq 0.05$

Table 3: Relation between differentiation and eosinophilic count

Eosinophilic count	Differentiation			H	p
	WD	MD	PD		
Non metastatic	(n = 13)	(n = 8)	(n = 4)		
Min. – Max.	6.0 –7.80	8.10 –10.0	10.3 –13.0		
Mean ± SD.	6.98 ±0.54	9.13 ±0.68	11.48 ±1.15	19.803*	<0.001*
Median	6.90	9.20	11.30		
Sig. bet. grps.	p_1=0.001*, p_2<0.001*, p_3=0.183				
Metastatic	(n = 8)	(n = 8)	(n = 9)		

Min. – Max.	5.4 –7.0	3.6 –4.7	2.5 –3.0		
Mean ± SD.	6.09 ±0.49	4.19 ±0.41	2.8 ±0.17	21.383*	<0.001*
Median	5.95	4.15	2.80		
Sig. bet. grps.	p_1=0.030*,p_2<0.001*,p_3=0.017*				

H: Kruskal Wallis test
p: p value for association between different categories
*: Statistically significant at $p \leq 0.05$

Table 4: Correlation between eosinophilia count and age in each group

	Eosinophilic count			
	Non metastatic		Metastatic	
	r_s	p	r_s	p
Age	-0.236	0.255	-0.015	0.942

r_s: Spearman coefficient

DISCUSSION

Oral cancer is a paramount health problem, causing high rates of mortality and morbidity, and responsible for 275,000 newly diagnosed cases and 128,000 fatalities annually worldwide (35). The oral cavity represents the 6th to the 9th most common site for cancer, according to the difference between countries and even the gender of patients (36). In Egypt, the incidence of oral cancer in 2015 was 0.9% in males and 0.75% in females (37). Oral cancer is a multistep process where after the early exposure to a carcinogen, a latent period of up to 25 years may precede the appearance of cancer (35). The prognosis of OSCC is frequently poor as a result of the late discovery of most lesions, after they have attained a large size. Consequently, emerges the importance of early diagnosis of cancer which enhances the five-year survival rate to 90% versus 20% in case of late diagnosis (37).

The present study was done in order to identify a feasible and economical method to assess and compare eosinophilic infiltration in both metastatic and non-metastatic OSCC using Congo red stain and its correlation to the tumor grade. Eosinophils are normal residents of oral lamina propria. Their number escalates with carcinogenesis starting with epithelial dysplasia. It could be an indication that the immune system is not functioning well if the number of eosinophils is very low. The rationale why this present study patients revealed the contrary, i.e. better survival with no eosinophils or medium TATE, can only be guessed. As it's known that cancer progress is not only reliant on cancer cells but also on the adjacent stroma (38).

Recently, it was proposed that eosinophils (EOS) can influence tumors directly and/or indirectly, and it looks as if that the microenvironment of the tumor might provide additional indications for degranulation of eosinophils and elimination of the tumor. The anti-tumor impact of EOS, is associated with the release of cytotoxic proteins, e.g. ECP, major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), which has been linked with tumor cell apoptosis (39).

Albeit a potential part for EOS in anti-tumor activity, various researchers implied the probability that the EOS, recruited to tumor sites, support angiogenesis also are component of the host connective tissue response to the tissue injury generated by the developing tumor (13).

Many studies were performed to determine the role of eosinophilic count (EC) in carcinomas of oropharyngeal carcinomas. However, only few have been performed for OSCC. Furthermore, many parameters have been studied in relation to EC (40).

In the current study, the eosinophilic count was obtained from histological fields of 50 cases stained by Congo red stain. The relation between age, gender, site of the tumor and differentiation of the tumor was tested.

In relation to the gender, the metastatic tumors in females showed higher eosinophilic count compared to the males.

Correlating the EC to the degree of tissue differentiation, it was found that eosinophils acts differently in the metastatic and non-metastatic tumors. The EC increases with the decrease of differentiation in the non-metastatic tumors. However, the opposite was observed in the metastatic group where the eosinophilic count decreased significantly with the decrease in differentiation.

The current study demonstrated a higher count of EC in non-metastatic OSCC compared to metastatic ones, which agrees with Ohashi *et al.* and Ishibashi *et al*. Findings indicated the importance of EC in the biological behavior of OSCC. This implies a correlation between EC and the biological behavior of tumor (19, 41).

Dorta *et al* demonstrated the effect of tissue eosinophilia on the prognosis of OSCC patients. All the samples were explored throughout the full depth, encompassing tumor stroma and malignant cells, embracing a total area of 1.32mm^2 per tumor, registering 75 random microscopic fields. Better prognosis was found in patients with higher numbers of tumor-associated EOS than in patients with small or intermediate totals. Although this concurs with the current study but still we cannot predict the prognosis for our results (16).

TATE was shown by multivariate analysis to be an objective prognostic element when supplemented by age, gender, alcohol or tobacco consumption, tumor site, T and N clinical stage and vascular embolization. Accordingly, the researchers proposed that EOS might have a protecting part against tumor advancement (13).

Interestingly, others researchers also discovered a substantial connection between high-grade tissue eosinophilia, rareness of remote metastasis and favorable prognosis in head and neck OSCC (42, 43). The outcomes of these studies have to be clarified with concern as samples from a few separate head and neck locations, along with broadly erratic prognosis, were incorporated in the study. Furthermore, the limit for TATE

diagnosis was lesser than in other researches and the assessment of poor or good result was done after a fairly small follow-up interval of 2 years (14).

As regards of the role of TATE, several researches were directed by Goldsmith et al. (1987), Goldsmith et al. (1992). All these researches imply that escalated number of TATE is linked with antitumoral role and displays good prognosis (42, 43). These are in concordance with the present research, which also exhibited an escalation in TATE in non-metastatic cases than metastatic cases, consequently backing good prognosis.

On the other hand, it was suggested that tissue eosinophils take part in OSCC tumor-promoting (44). Studies showed that patients with high eosinophil indices had a statistically significant lower survival than those with lower eosinophil indices (45).

It was also found that TATE had no prognostic value in OSCC and suggested that strong TATE appears to indicate the stromal invasion of the OSCCs which occurs in advanced clinical stage (44).

CONCLUSIONS

Referring to the limitations of the present study, we conclude that the Eosinophilic count is a strong indicator to determine the stage and degree of differentiation of the tumors.

STATEMENT OF CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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