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Original Article

A histochemical study of tissue eosinophilia in oral squamous cell carcinoma using Congo red staining

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ABSTRACT

Background: Tumor associated tissue eosinophilia is believed to play a significant role in the biological behavior of the carcinoma. Eosinophil infiltrate in association with the head and neck squamous cell carcinoma (SCC) have been reviewed from time-to-time. The significance of such an association has been variably thought to be either a potential diagnostic tool for stromal invasion or as a prognostic indicator. We aimed to evaluate the efficacy of Congo red staining to differentiate eosinophils in the inflammatory infiltrate in oral squamous cell carcinoma (OSCC) and whether this eosinophilia is associated with the histologic grading in OSCC.

Materials and Methods: The eosinophil infiltration in hematoxylin and eosin (H and E) and Congo red stained sections of 50 biopsies of OSCC were examined. The eosinophil distribution was quantitatively evaluated in both sections as either diffuse or focal and scored as mild, moderate and severe grades. The average number of eosinophils obtained in OSCC stained by H and E and Congo red were then statistically compared by univariate analysis carried out using Student's *t*-test. $P < 0.01$ was used to determine the statistical significance.

Results: In the OSCC, the eosinophil infiltration was seen in 43 out of 50 (86%) cases. The elevation of eosinophil count was more diffuse than focal (36 vs. 7 [83.72 vs. 16.27%]). The staining efficacy of Congo red stain over H and E stain to differentiate eosinophils was excellent and found to be statistically significant ($P < 0.01$). No significant correlation was found with the eosinophil infiltration and the histologic grades of OSCC.

Conclusion: Our study showed a strong infiltration of eosinophils in OSCC though no significant correlation was found with the eosinophil infiltration and the histologic grades of OSCC. Congo red staining showed a high sensitivity in staining eosinophils over routine H and E. This staining technique could therefore provide an adjunct to routine H and E in evaluating eosinophils in dysplasia and OSCC cases.

Key Words: Congo red, eosinophils, hematoxylin and eosin, oral squamous cell carcinoma, tumor associated tissue eosinophilia

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) constitutes approximately 90% of all oral malignancies. It is the fifth most common cancer world-wide and in India, it is the most common cancer. In an epidemiological study conducted at Isfahan, Iran by Razavi and Sajadi, squamous cell carcinoma (SCC) was found to be the most common cancer with a frequency of 54.5%.[1] SCC is defined as a malignant neoplasm that is derived from or exhibits the morphologic features of squamous epithelium. It is often the end stage of a series of alterations in stratified squamous epithelium.

Despite many improvements in treatment over the past 30 years, little progress has been made in improving survival rates. Therefore, the prevention and any innovation that facilitates early detection of this neoplasm

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have the potential to improve survival and quality-of-life. The histopathological grade of the tumor is related to its biologic behavior. In addition to the histopathological features that can effect on its prognosis, many efforts have been carried out to find molecular markers that can predict its biologic behavior.[2]

As far as predicting prognosis is concerned, recently the role of heat shock proteins (HSP) is worth noting. HSPs are unique cytoprotective proteins that are thought to be the most ancient defense system in all living microorganisms of the earth. Among HSPs, HSP70 and HSP27 seem to have a strong association with cancer and many studies have found alteration of their expression level in different cancers. Prognostic value of HSP27 in OSCC is not clear as conflicting results have been reported. Deyhimi and Azmoudeh examined the relationship between the expression of HSP70 with oral SCC and no significant differences between the expression of this marker in normal tissue and oral cancer was observed.[2]

Evolution of OSCC is influenced by the host immune response cells (e.g., CD8+ T, CD4+ T, natural killer cells (NK), dendritic cells, macrophages and eosinophils).[3] In the head and neck SCCs, tumor cells attract monocytes and activate them to secrete angiogenic factors. In addition, macrophages produce cytokines that act on tumor cells and stimulate them to produce increased levels of interleukin-8 (IL-8) and vascular endothelial growth factor. In this context, Jahanshahi, Sabaghian. have conducted an immunohistochemical analysis of angiogenesis and mast cell density in OSCC and have not found any positive correlation between the values of mast cell density and micro vessel density in the OSCC group as compared to normal oral mucosa.[4]

In routine histopathology, it is difficult to differentiate between pre-invasive squamous neoplasia and squamous carcinoma in small biopsy specimens, when tissue is superficial or tangentially cut or obscured by a prominent inflammatory infiltrate. Even in larger resection specimens, the presence of invasion may sometimes be elusive if the invasive element lacks features of invasive squamous carcinoma. This differentiation between pre-invasive squamous neoplasia and squamous carcinoma is important because even though both have the same genetic aberrations, pre-invasive squamous neoplasias are potentially reversible after cessation or removal of an instigating factor like tobacco use.[5]

Tumor associated tissue eosinophilia (TATE) is characterized by the presence of eosinophils in the tumoral inflammatory infiltrate and has been recognized for almost 100 years in many carcinomas located in larynx, pharynx, lung, external genitalia, gastrointestinal tract and oral cavity.[6-8] In routine histopathology when such an association is documented it is regarded as non-specific microscopic feature of questionable importance.[8]

The eosinophils are considered as destructive effector leukocytes with cytotoxic activities and are also involved in tissue remodeling and in innate and acquired immunity response modulation. The eosinophils release various substances such as, eosinophil cationic protein, major basic protein, eosinophil peroxidise, eosinophil-derived neurotoxin, IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18, interferon-γ, tumor necrosis factor-α, transforming growth factor (TGF)-α, TGF-β, chemokines, etc.[9] These substances may cause cell death and induction of inflammatory symptoms as well as contribute to tumor progression or regulation [Figure 1]. In OSCC, the role of eosinophils is unclear and they have been associated with good as well as bad prognosis.[9]

In routine H and E staining, the presence of eosinophils might not be demonstrated easily as it may be overshadowed by inflammatory cell infiltrate or eosinophils may assume abnormal morphology. We tried to find out if Congo red stain could be used easily in routine histopathology to demonstrate eosinophils. Thus, we performed this study aiming to demonstrate TATE in different grades of OSCC and to compare routine H and E and Congo red staining efficacy in doing so.

MATERIALS AND METHODS

Our study consisted of 50 incisional biopsies of OSCC which were retrieved from Oral Pathology

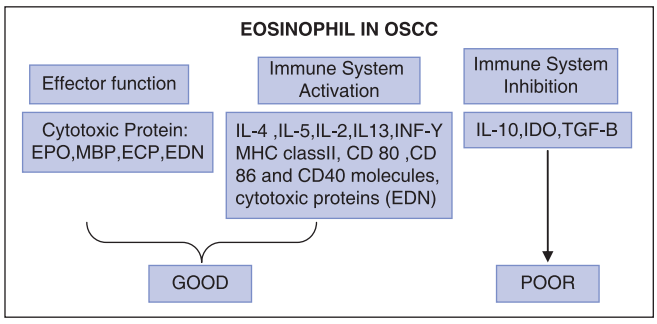

Figure 1: Role of eosinophils in oral squamous cell carcinoma

Department registry of Vasantdada Patil Dental College and Hospital, Sangli from the year 2009 to 2011. Out of 50 cases, 36 were well-differentiated SCC, 12 moderately differentiated SCC and 2 poorly differentiated SCC. From the paraffin embedded blocks of selected cases 4 μm thickness sections were cut and stained with conventional H and E and Congo red stain. For each section, the high-power field (HPF) (×40 ocular objective lens) with a maximum number of eosinophils was identified and recorded. Then, the eosinophils in the adjacent nine contiguous HPF were counted, added to those in the first and recorded as eos/10 hpf. Only nucleated cells with intensely red cytoplasmic granules were accepted as eosinophils and care was taken to exclude red blood cells with superimposed mononuclear and polymorphonuclear inflammatory cells. Those that were confined to lymphovascular spaces were excluded.

Statistical methods

Elevated eosinophils in the Congo red stained sections were defined and categorized as: Focally and mildly elevated (0-4 eos/10 hpf), focally and moderately elevated (5-9 eos/10 hpf), focally and severely elevated (≥10/10 hpf), diffusely and mildly elevated (0-4 eos/10 hpf), diffusely and moderately elevated (5-19 eos/10 hpf) and diffusely and severely elevated (>20/10 hpf). The average no of eosinophils obtained in OSCC stained by H and E and Congo red were then statistically compared by Univariate analysis carried out using student's *t*-test. $P < 0.01$ was used to determine the statistical significance.

Table 1: Eosinophilia in OSCC

OSCC (total no.)	Eosinophilia present	Eosinophilia absent
50	43 (86%)	7 (14%)

OSCC: Oral squamous cell carcinoma

RESULTS

A total of 50 incisional biopsy sections of OSCC were evaluated. Among the 50 biopsy sections, 36 were well-differentiated SCC, 12 were moderately differentiated SCC and 2 were poorly differentiated SCC. Among the total 50 cases, tissue eosinophilia was present in 43 cases (86%) [Table 1].

As shown in Table 2, 36 (83.72%) cases were found to have elevated diffuse eosinophilic infiltration out of which 21 (48.83%) were severely elevated, 13 (30.23%) were moderately elevated and 2 (4.65%) were mildly elevated. Only 7 (16.27%) cases showed focal eosinophilic infiltration out of which 4 were severely elevated and 3 were mildly elevated. Interestingly the eosinophils were found intimately associated with tumor cells or with a strong lymphoplasmatic cell infiltration.

In Congo red staining, eosinophils were easily recognized as compared with H and E staining, by a brightly red-stained cytoplasm with the background of other tissue structures stained in dark blue [Figures 2-4].

The TATE could be demonstrated very effectively by Congo red staining ($P = 0.0005$) as compared with H and E staining [Figure 5a-c and Table 3] and the results are statistically significant. There was no association between elevated tissue eosinophils and overall inflammatory response of the stroma in the specimens studied. As the distribution of OSCC cases according to histologic grades was unequal and not consistent, no correlation was noted between the eosinophil infiltration and histologic grades of OSCC.

DISCUSSION

Leighton defined TATE as the "tumoral infiltration by eosinophils not related to the presence of necrosis and/or ulceration;" although, the definition was arbitrary and poorly defined.[10]

Table 2: Distribution of eosinophils in OSCC

Distribution OSCC type	Focal			Diffuse		
	Mildly elevated	Moderately elevated	Severely elevated	Mildlyel evated	Moderately elevated	Severely elevated
Well-differentiated OSCC (*n*=30)	1	0	4	2	8	15
Moderately differentiated OSCC (*n*=11)	2	0	0	0	4	5
Poorly differentiated OSCC (*n*=2)	0	0	0	0	1	1

OSCC: Oral squamous cell carcinoma

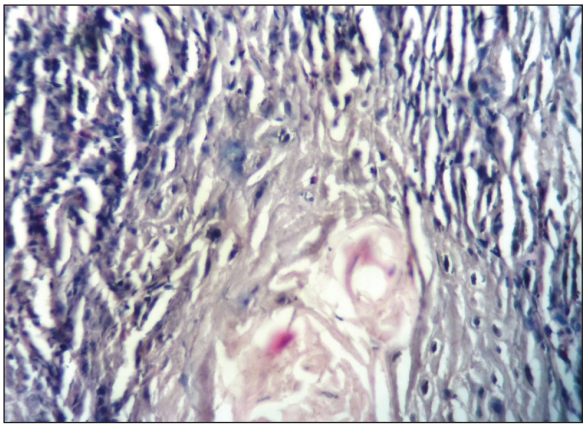

Figure 2a: Well-differentiated squamous cell carcinoma; H and E (×40)

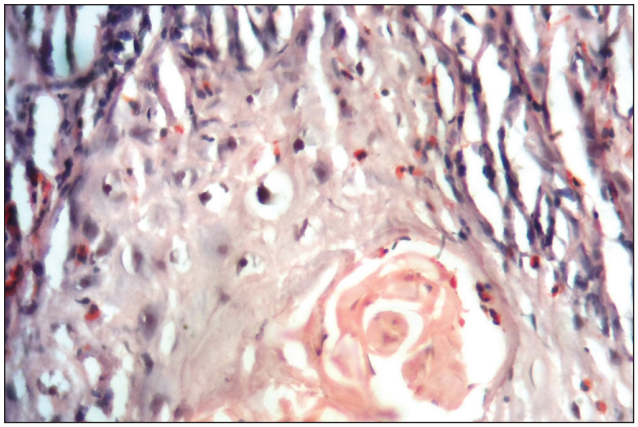

Figure 2b: Well-differentiated squamous cell carcinoma; Congo red (×40)

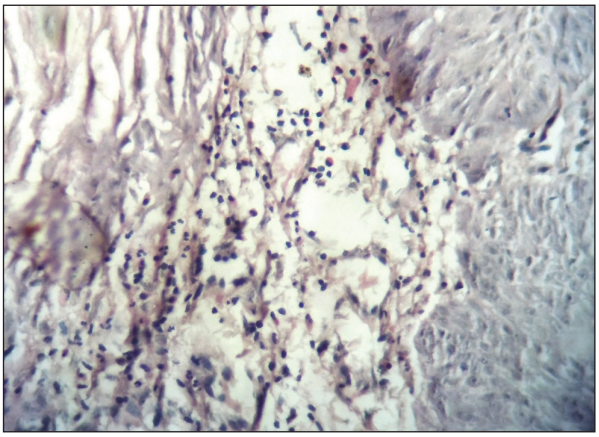

Figure 3a: Moderately differentiated squamous cell carcinoma; H and E (×40)

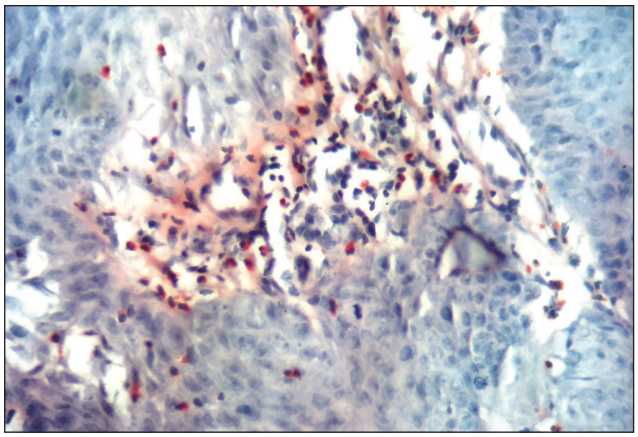

Figure 3b: Moderately differentiated squamous cell carcinoma; Congo red (×40)

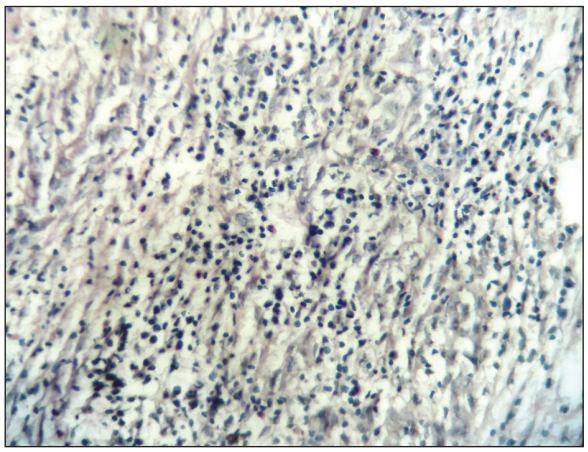

Figure 4a: Poorly differentiated squamous cell carcinoma; H and E (×40)

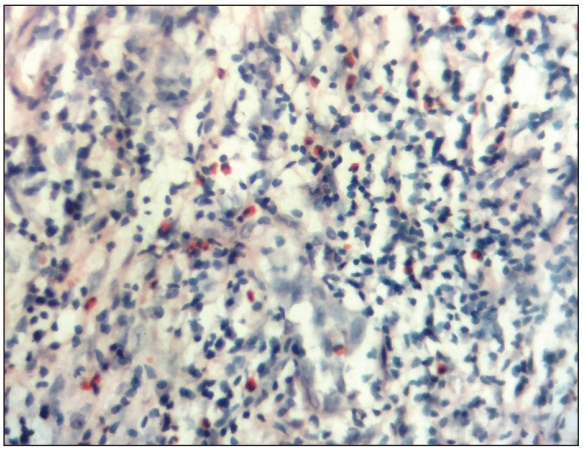

Figure 4b: Poorly differentiated squamous cell carcinoma; Congo red (×40)

The role of TATE in OSCC is unclear. The studies by Goldsmith *et al*., in 1987,[11] and in 1992;[12] Thompson *et al*., in 1994;[13] Nielsen *et al*., in 1999;[14] Fernández-Aceñero *et al*., in 2000;[15] Dorta *et al*., in 2002[7] have associated TATE with a favorable prognosis while the studies by Horiuchi *et al*., in 1993;[16] van Driel *et al*., in 1999;[17] Wong *et al*., in 1999[18] with an unfavorable prognosis and some

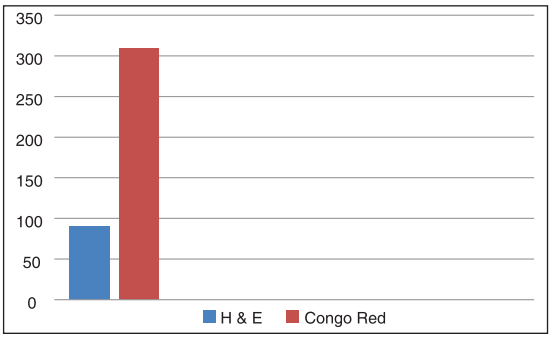

Figure 5a: Comparison of tissue eosinophils in oral squamous cell carcinoma stained with H and E and Congo red in welldifferentiated squamous cell carcinoma

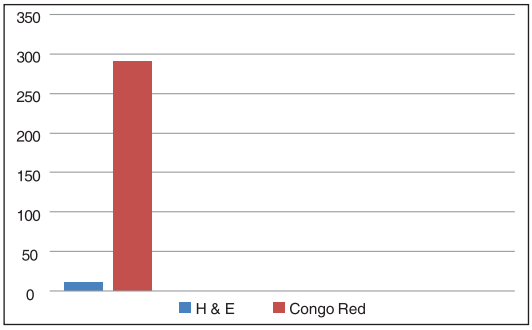

Figure 5b: Comparison of tissue eosinophils in oral squamous cell carcinoma stained with H and E and Congo red in moderately differentiated squamous cell carcinoma

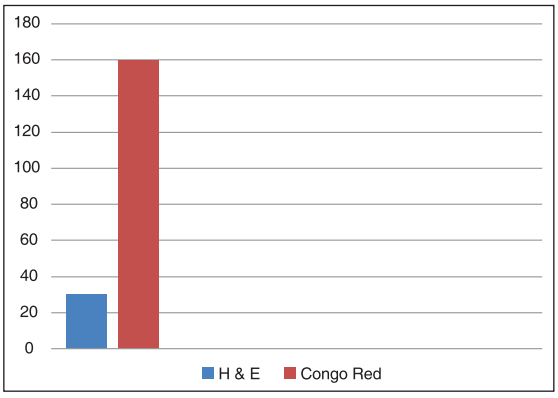

Figure 5c: Comparison of tissue eosinophils in oral squamous cell carcinoma stained with H and E and Congo red in poorly differentiated squamous cell carcinoma

Table 3: Comparison of eosinophil staining in OSCC by H and E and congo red

Eosinophils in OSCC			
Group	**Mean**	**Standard deviation**	***P* value (*t*-test)**
Congo red	75.7059	99.0964	*P*=0.0005
Hematoxylin and eosin	12.8529	16.1135	

OSCC: Oral squamous cell carcinoma

studies like Looi, in 1987;[19] Sassler *et al*., in 1995[20] have shown no influence on the prognosis.

In the head and neck SCC, it has been reported that the presence of tissue eosinophils ranges between 22% and 89%.[5] In our study, we observed that TATE was present in 86% (43 out of 50) cases, which is in accordance with other studies.

Deron *et al*. in their study to evaluate TATE kept the cut-off of eosinophil infiltration at 2 eos/hpf.[21] Goldsmith *et al*., in their study, segregated eosinophilic infiltration within 4 categories of progressive increase, the highest being categorized as 4+, that is, more than 30 eos/hpf.[11,12] Said *et al*., in their study, evaluated 87 cases of invasive SCC and pre-invasive squamous neoplasia and segregated the elevated eosinophils as: Focally and moderately elevated (5-9 eos/hpf), focally and markedly increased (>10/hpf), diffusely and moderately elevated (5-19 eos/10 hpf) and diffusely and markedly increased (>20/10 hpf).[5]

In our study, we categorized and graded the elevated eosinophil count as focal and mildly elevated (0-4 eos/10 hpf), focal and moderately elevated (5-9 eos/10 hpf), focal and severely elevated (≥10/10 hpf), diffusely and mildly elevated (0-4 eos/10 hpf), diffusely and moderately elevated (5-19 eos/10 hpf) and diffusely and severely elevated (>20/10 hpf). In our study, most of the OSCC cases had diffuse and severe infiltration of eosinophils and they were found intimately associated with tumor cells or with a strong lymphocytic and plasma cell infiltration.

Although intact eosinophils are usually easily identified in routine H and E sections, sometimes these granulocytes assume an uncommon morphology especially in fibrous tissue and inflammatory infiltrate making their recognition in routinely stained sections very difficult. We tried to determine if Congo red stain could better demonstrate TATE. TATE eosinophilia was demonstrated very effectively by Congo red staining (P = 0.0005) as compared to H and E staining [Figure 5a-c and Table 3] and the results are statistically significant. In Congo red staining, eosinophils were easily recognized by a brightly red-stained cytoplasm with the background of other tissue structures stained in dark blue [Figures 2-4]. Thus, the Congo red stain can be used to easily identify eosinophils and it is particularly important in OSCC wherein the incisional biopsy specimens are very tiny and stromal invasion cannot be easily assessed in such biopsies.

There was no association of elevated tissue eosinophils with overall inflammatory response of the stroma in the specimens studied. As the distribution of OSCC cases according to histologic grades was unequal and not consistent, no correlation was noted between the eosinophilic infiltration and the histologic grades of same.

CONCLUSION

In conclusion, our study showed a strong infiltration of eosinophils in OSCC. Congo red staining showed a high sensitivity in staining eosinophils over routine H and E. This staining technique could therefore provide an adjunct to routine H and E in evaluating eosinophils in OSCC cases and could be used as an additional morphological feature that can be mentioned in the biopsy report.

Evidence of eosinophil infiltration in dysplastic cases should prompt thorough evaluation for invasiveness, especially when evidence of invasion is absent or suspected when biopsy specimens are too small. Whether TATE is a useful prognostic indicator can be evaluated by studies carried out on long-term follow-up of OSCC cases.

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