Comparative evaluation of eosinophils in normal mucosa, dysplastic mucosa and oral squamous cell carcinoma with hematoxylin-eosin, Congo red, and EMR1 immunohistochemical staining techniques

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Abstract

Background: Oral squamous cell carcinoma is the most common malignant lesion of the oral cavity, and it involves various molecular mechanisms. The development of oral squamous cell carcinoma is influenced by the host immune cells, such as eosinophils. The present study was conducted to compare the presence of eosinophils in normal mucosa, dysplastic mucosa, and oral squamous cell carcinoma by -hematoxylin- eosin staining, Congo red staining, and epidermal growth factor-like (EGF-like) module containing a mucin–like hormone receptor1 (EMR1) immunohistochemical marker.

Methods: In this cross-sectional study, 60 paraffinized samples were selected, consisting of 20 normal mucosae, 20 dysplastic mucosae, and 20 squamous cell carcinoma samples. After confirmation of the diagnosis, the mean number of eosinophils was evaluated by hematoxylin-eosin, Congo red, and immunohystochemical staining techniques. The data were analyzed by SPSS-10 software using the Kruskal-Wallis and Friedman tests.

Results: The results showed that the number of eosinophils in dysplastic mucosa was significantly higher than the number in normal mucosa, and the number of eosinophils in squamous cell carcinoma was significantly higher than the number in dysplastic mucosa in all staining techniques (p<0.001). Moreover, the comparison of staining techniques showed a significantly higher number of eosinophils in EMR1immunohistochemicalmarker than were observed when Congo red and hematoxylin - eosin (H&E) staining techniques were used (p<0.001).

Conclusion: It can be argued that eosinophil contributes to the identification of lesions that have a higher potential of malignant transformation. Moreover, eosinophil can be suggested as an indicator in the differentiation of oral lesions in cases with borderline diagnosis and in targeted molecular therapy.

Keywords: Congo red, Dysplastic, EMR1, Eosinophil, Normal mucosa, Squamous cell carcinoma

1. Introduction

Oral squamous cell carcinoma (OSCC) comprises approximately 90% of all head and neck cancers, especially in young people (1, 2), and its occurrence is increasing in the developing countries (3). In an epidemiological study conducted in Isfahan, Iran, oral squamous cell carcinoma (OSCC) was found to be the most common cancer, and it

occurred most frequently in the gingiva (4). OSCC is considered with a multifactorial etiology, not a single etiology (5). The etiology and pathogenesis of head and neck SCC are influenced by environmental factors, but the main causative mechanism has yet to be identified (6). The prognosis of oral SCC is poor even with combined methods of surgery, radiotherapy, and chemotherapy. The 5-year survival for patients is about 40% (7). Despite the extensive research on the pathogenesis and treatment of this tumor, no significant breakthroughs have occurred to date (8). SCC arises from dysplastic epithelial cells and invades the underlying connective tissue in the forms of cords and islands (9).

Dysplasia is a term that means pathological growth that changes oral mucosal tissues from normal to dysplastic and consequently SCC occurs gradually over time. However, the dysplastic changes that occur are not always cancerous. Thus, it is necessary to identify the factors that affect these changes to gain a better understanding of the molecular mechanisms and to identify the potential of oncogenes that produce malignant changes in oral tissues. This recognition may lead to new therapeutic methods (treatment of target molecules) in patients with OSCC (10-13). Understanding the molecular mechanisms contributes to early diagnosis, the correction of dysplastic lesions, and the prevention of possible changes of these lesions into OSCC. Early diagnosis of OSCC can lengthen the life of the patient and dramatically reduce the high rate of mortality (14). It is possible for recursion to occur after the elimination of background factors (15).

Studies have shown that the development of OSCC is influenced by host immune cells, LTCD8+, LTCD4+, NK, macrophages, dendritic cells, and eosinophils (16). Eosinophils constitute about 5% of white blood cells and have two-lobed nuclei. The cytoplasm of these cells contains abundant granules. Eosinophils secrete various materials, such as cationic protein, peroxidase, chemokines, IFNγ, TNFα, TGFα, TGFβ, and, some sub-types of interleukins. These substances cause cells to die and induce inflammatory symptoms due to the development of tumors (17). There are questions about the role of eosinophils in OSCC, and they concern whether there is any relationship between the increase of tissue eosinophils and dysplastic changes and the occurrence of OSCC. In the eosin-hematoxylin staining technique, all inflammatory cells are stained uniformly, and it is difficult to distinguish eosinophils from other inflammatory cells. In the Congo red staining method, eosinophils with shiny red cytoplasm can be analyzed more effectively than they can be analyzed in the hematoxylin and eosin staining technique (18). In addition, the role of these inflammatory cells can be analyzed more accurately by the EMR1 immunohistochemical marker, which is a specific receptor for eosinophils (19).

Human epidermal growth factor-like (EGF-like) modules that contain mucin–like hormone receptor1 (EMR1) are superficial receptors with unknown performance that belong to the family of the G protein-coupled receptors (GPCR). These receptors are so named because they have seven transmembrane alpha helices. They constitute the largest family of plasma membrane receptors, 1500 of which have been identified to date. They transmit messages to the cells through the proteins connected to G proteins. A large number of ligands, such as histamine and serotonin, deliver messages via these receptors. Ligand binding causes changes in the spatial form of the receptor, thereby inducing its activation and allows for reactions with many G proteins. G proteins are activated by changing the GDP that exists in inactive proteins into GTP in active proteins (10). This superficial receptor is specific to eosinophils (19). The aim of this study was to test the role of eosinophils in dysplastic and cancerous lesions and to determine the valuable criterion of the sensitivity of the Congo red staining technique compared to the sensitivity of eosin-hematoxylin staining and immunohistochemical staining.

2. Materials and methods
2.1. Study design and setting
This was an analytical-descriptive study conducted on patients with head and neck SCC in the Isfahan School of Dentistry and Alzahra and Kashani Hospitals. This study was conducted in the Isfahan School of Dentistry, Department of Oral Pathology during 2014 and 2015. Sixty paraffin embedded blocks, including 20 normal mucosae, 20 dysplastic mucosae (7 mild, 7 moderate, and 6 severe mucosae), and 20 squamous cell carcinoma (6 poorly differentiated, 7 moderately differentiated, and 7 well differentiated) related to the past three decades (1985-2015) were collected from the archives of the Pathology Department at the Isfahan School of Dentistry and Alzahra and Kashani Hospitals in Isfahan, Iran. The normal mucosas were obtained from the patients who had undergone a dental implant. The required sample size was calculated using the formula "$n = (Z_{1-\alpha/2}+Z_{1-\beta})2(\delta_1^2-\delta_2^2)/d^2$"; Where: $\alpha=0.05$, $Z_{1-\alpha/2}=1.96$, $Z_{1-\beta}=0.84$, $\beta=0.2$, $\delta_1\delta_2\approx11$, and $n=20$.

2.2. Selection criteria
The inclusion criteria for this study were specimens that 1) had sufficient tissue and 2) had little inflammation. The exclusion criteria were specimens that showed inflammation or any pathologic findings.

2.3. Data collection
Four-micrometer sections were prepared from the samples for histopathological evaluation and confirmation of previous diagnoses of the samples. After confirmation, the sections were stained by the immunohistochemical staining technique (EMR1 marker, Santa Cruz, USA) and the Congo red staining techniques. For EMR1 immunohistochemical evaluation, 4-5-μm tissue sections were prepared. Deparaffinization of the samples was performed by immersing them in xylene. The sections were hydrated by descending ethanol, and, then, they were incubated in proximity of 5% hydrogen peroxide for 30 minutes to terminate the activity of the endogenous peroxide. Antigen retrieval was performed for 10 minutes in a 0.01 M sodium citrate buffer in a 700-W microwave oven, and, then, the sections were incubated in normal saline for 30 minutes.

The primary antibody (Lab Vision EMR1-Ab-1), at a concentration of 1 mg/ml, was added for one night at a temperature of 4 °C. Then, the samples were incubated with the secondary antibody (Singnet lab) for 30 minutes and was placed in streptavidin peroxidase for 1 hr. Chromogenic reaction was performed by diaminobenzidine, and opposite staining was done by hematoxylin. Phosphate-buffered saline (PBS) was used in the negative controls and breast adenocarcinoma was used for positive controls. For Congo red staining, a 3-4 μ section was prepared and differentiated in 1% ethanol. The samples were washed with running water for 1 min and placed in 1% Congo red (Merck Germany) aqueous solution for 20-30 min. Then, the samples were placed in saturated lithium carbonate for 15 min, after which they were placed in 80% ethanol. Dehydration, clearing, and pasting were performed. All of the samples that were stained by the IHC and Congo red staining techniques were evaluated separately by two pathologists using an optical microscope (Olympus BX41TF, Tokyo, Japan) with 400× magnification. To this end, 10 non-overlapping fields were selected, and the eosinophils in the fields were counted.

2.4. Research ethics
Our study protocol was approved by the Isfahan Research and Ethics Committee (Ethics number approved: 393384). The normal specimens were prepared using the mucosa that was removed during the implant procedure.

2.5. Statistical analysis
Data were analyzed by SPSS version 22 using the Kruskal-Wallis and Friedman tests because, according to the one-sample Kolmogorov-Smirnov test, a normal distribution was not observed in some groups. $P < 0.001$ was considered significant.

3. Results
The Congo red staining method showed the presence of eosinophils with red cytoplasm in epithelial cells and connective tissue of the samples, and a higher concentration of eosinophils was observed in the invasion front (Figure 1). EMR1 immunohistochemical staining technique indicated the presence of eosinophils with brown cytoplasm in epithelial cells and connective tissue (Figure 2). The number of eosinophils was obtained in all samples by three staining techniques (Table 1).

Table 1. Mean and standard deviation of eosinophils in normal, dysplastic, and squamous cell carcinoma mucosa obtained by three staining techniques

Tissue type		Congo red	Immunohistochemical	Hematoxylin-Eosin
Normal		0.65±1.03	1±1.02	0.25±0.44
Dysplastic	Mild	2.57±2.07	3.28±2.28	1.14±1.06
	Moderate	6.14±2.19	6.57±2.69	2.85±1.34
	Severe	15.8±8.07	17.4±10.35	8.2±2.49
SCC*	Well differentiation	8±1.52	12.58±4.35	5.14±0.89
	Moderate differentiation	31.28±31.23	44.14±38.23	16±7.32
	Poor differentiation	100±0	110±0	32.82±11.26

* Squamous cell carcinoma
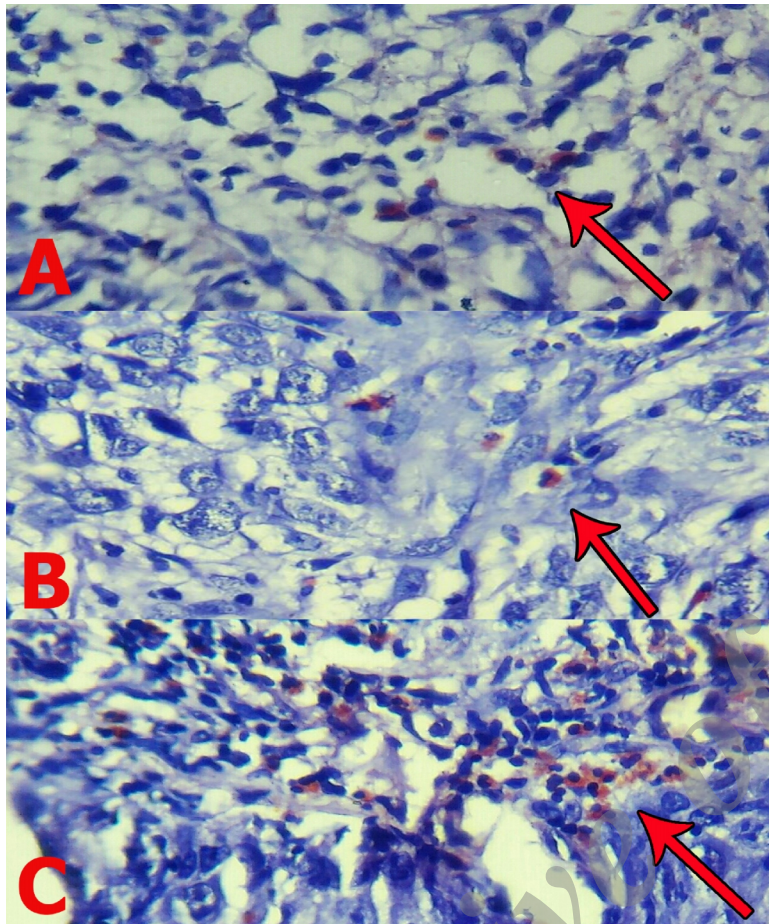
Figure 1: Congo red staining In dysplastic mucosa (A), SCC (B), and early Invasive SCC (C)
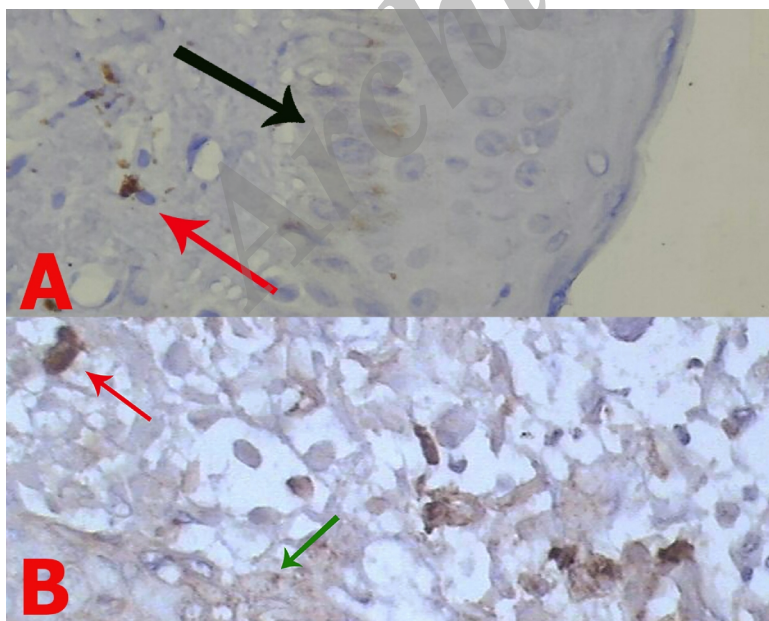
Figure 2: EMR1 expression In dysplastic mucosa (A), and SCC (B). Red arrows show eosinophils; green arrow shows carcinomatos epithelial nest of SCC; black arrow shows dysplastic epithelium.

The results of the Kruskal-Wallis and Mann-Whitney tests indicated a significantly higher mean of eosinophils in the dysplastic mucosa than in normal mucosa ($p<0.001$) and a significantly higher mean of eosinophils in squamous cell carcinoma than in normal mucosa ($p<0.001$). Further, the results of the comparison of dysplastic mucosa and squamous cell carcinoma showed a higher mean of eosinophils for squamous cell carcinoma ($p<0.001$).

The findings of the Friedman and Wilcoxon tests to compare the staining techniques showed that the Congo red staining method revealed a significantly higher number of eosinophils than H&E staining ($p<0.001$). Moreover, the EMR1 immunohistochemical staining showed a higher number of eosinophils than either the Congo red or the H&E staining techniques ($p<0.001$). According to receiver operating characteristic-curve (ROC-CURVE) it seems that in terms of sensitivity, the number of eosinophils >5 (sensitivity=0.684, specifity=0.050) showed normal-dysplasticborderline, and the number of eosinophils >15 (sensitivity=0.619, specifity=0.053) was indicative of the borderline of dysplastic-squamous cell carcinoma (Figure 3, 4).
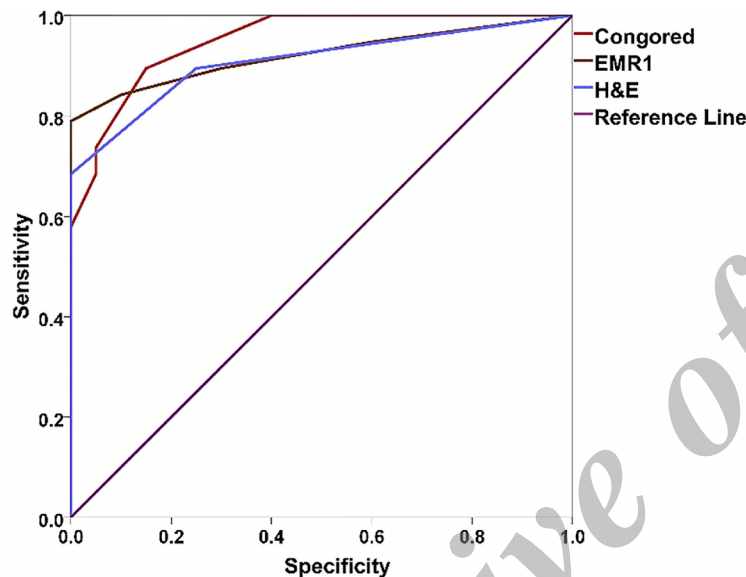

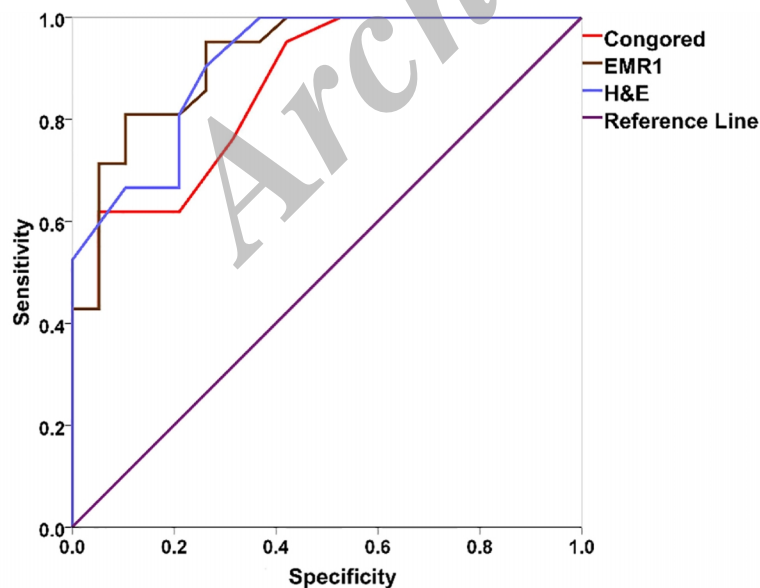**Figure 3.** ROC-CURVE of normal and dysplastic mucosa

Figure 4. ROC-CURVE of dysplastic and squamous cell carcinoma mucosa

4. Discussion

Early diagnosis and prediction of tumor behavior are the most important issues associated with the treatment of patients with OSCC and pre-malignant lesions. Moreover, dysplastic changing of oral mucosa tissues from normal to dysplastic and consequently to SCC occurs over time. There is an increasing need to gain a better understanding of the molecular basis of oral carcinoma and to diagnose the potential of effective oncogenes for early diagnosis and correction of dysplastic lesions, which may lengthen the patient's life and reduce the mortality rate associated with this disease. The development of OSCC is underpinned by the host immune cells, such as eosinophils (11-13).

Eosinophils secrete various materials, including eotaxins, TGFα, TGFβ, and IL (17). These secretory substances, in addition to inducing inflammatory symptoms as a result of development, may interfere with enhancing the development. For instance, TGFα is a stimulant of mitogen for different cells, including epithelial cells. TGFβ also is a proliferative factor for keratinocytes and fibroblasts, and it has a significant role in the formation of fibrosis and chronic inflammatory conditions because it reduces proteinase (10). Accordingly, it can be assumed that eosinophil is a contributing factor to the development of tumors. In the present study, the changing trend of eosinophils from normal mucosa to dysplastic mucosa and consequently to SCC was investigated by three staining methods, which indicated the increasing number of eosinophils in this trend. This is in line with the results of a study by Said in 2005 that showed the infiltration of eosinophils in the head and neck SCC (15). Also, the findings of Falconieri's research indicated that eosinophils were increased in pharyngeal cancers in the oral cavity (20). The results of a study by Lorena indicated that eosinophils had a significant role of in the development and survival of tumors by producing a substance called eotaxin (21).

IL5 is the most potent activator of eosinophils. By inhibiting the production of IL5, one study reported the shrinkage of tumors, which was indicative of the effective role of eosinophils in the development of tumors (22). The results of the present study were in agreement with these earlier findings in that the number of eosinophils increased in the dysplastic mucosa to a greater extent than in normal mucosa, which also occurred in SCC compared with dysplastic mucosa. The trend of the number of eosinophils in dysplastic development increased from mild to moderate to severe. In addition, in the present study, the number of eosinophils increased in three levels of SCC with well, moderate, and poor differentiation, which was in agreement with the results of Wong's study. Moreover, the results of the current study regarding the analysis of eosinophils in early-concentrated SCCs showed infiltration of eosinophils in the invaded region, which was in agreement with the findings of Falconieri's study, which indicated more invasion in connective tissue for OSCCs enriched with an infiltration of eosinophils (20). Furthermore, the results obtained in the current study were in agreement with the findings of Tostes' study in 2009 that investigated OSCC in different stages and reported the highest concentration of eosinophils in the areas where SCC invaded the underlying connective tissue (23). Other secretory cytokines of eosinophils play a role as well; for example, TGFβ inhibits the production of metalloproteinase (MMP) and consequently prevents the development of tumors, and IL4 prevents angiogenesis and tumor growth (10). These factors may explain the difference in the results obtained in the present study compared with those reported in Fernandez's study, which showed that all OSCC patients with similar treatment had a higher 5-year survival through frequent infiltration of eosinophil (24) as well as other findings that reported prevention of tumor development by eosinophils (25).

The different results found in various studies (26-28) that reported no correlation between eosinophils and tumor development and prognosis and provided insufficient information about the role of eosinophils in OSCC may have resulted from the difficulty in identifying and diagnosing eosinophils among other inflammatory cells by the common H&E staining technique. For better diagnosis of eosinophils in this study, the Congo red staining technique was used, and eosinophils with shiny red cytoplasm were analyzed more effectively than could have been done using the H&E staining method. This was in line with the results of a study conducted by Joshi in 2013 in which the incidence of eosinophils in OSCC was analyzed by both the H&E and Congo red staining techniques (18). Also, according to Haman's study, EMR1 immunohistochemical staining was specific to eosinophils (19). EMR1 was used in this study, and the results showed that eosinophils in OSCC could be analyzed more effectively than was the case using the H&E and Congo red staining techniques. In OSCC, it was shown that 91% of the EMR1 changes were explained by Congo red staining; therefore Congo red can be used with acceptable sensitivity and specifity. This staining technique is more accessible and more cost-effective than IHC by EMR1, and it is suggested for use in further investigations. Based on the ROC-CURVE, it seems that, in terms of sensitivity, the increasing number of eosinophils>5 shows normal-dysplastic mucosa borderline, and the increase of eosinophils>15 is indicative of

borderline dysplastic-squamous cell carcinoma. The above results can be considered as supplementary criteria along with other criteria for the diagnosis of dysplastic and squamous cell carcinoma in mucosa.

5. Conclusions
The number of eosinophils progressively increased from normal mucosa to dysplastic and squamous cell carcinoma mucosa. Also, it increased from mild to severe at different levels of dysplastic mucosa and from well differentiation to poor differentiation in squamous cell carcinoma. Accordingly, eosinophils can be regarded as an indicator of a developing malignancy along with other indicators, and they possibly can be used to develop the prognosis for the disease. In addition, the higher number of eosinophils in the EMR1 marker than in the Congo red and in H&E is probably indicative of the point at which anti-EMR1 can be used as a target molecule in the treatment of patients with squamous cell carcinoma and eosinophilic disorders. Furthermore, given the low cost of the Congo red staining technique, which provides acceptable sensitivity and specificity, it can be used for better diagnosis of eosinophils and to acquire a better understanding of their relationship with carcinogenesis.

Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
All of authors contributed to this project and article equally. All of authors read and approved the final manuscript.

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