Journal section: Oral Medicine and Pathology
Publication Types: Research

Expression of pro-inflammatory protein, iNOS, VEGF and COX-2 in Oral Squamous Cell Carcinoma (OSCC), relationship with angiogenesis and their clinico-pathological correlation

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Sappayatosok K, Maneerat Y, Swasdison S, Viriyavejakul P, Dhanuthai K, Zwang J, Chaisri U. Expression of pro-inflammatory protein, iNOS, VEGF and COX-2 in Oral Squamous Cell Carcinoma (OSCC), relationship with angiogenesis and their clinico-pathological correlation. Med Oral Patol Oral Cir Bucal. 2009 Jul 1;14 (7):E319-24.
http://www.medicinaoral.com/medoralfree01/v14i7/medoralv14i7p319.pdf

Received: 06/08/2008
Accepted: 01/10/2008

Article Number: 5123658892 http://www.medicinaoral.com/

eMail: medicina@medicinaoral.com
Indexed in:
-SCI EXPANDED
-JOURNAL CITATION REPORTS
-Index Medicus / MEDLINE / PubMed
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Abstract

One main etiology for oral squamous cell carcinoma (OSCC) is inflammation. Inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) are the important molecules showing close relation to not only inflammation but also carcinogenesis and angiogenesis. Angiogenesis is defined as the formation of new blood vessels from existing vasculature. It is necessary for tumor growth and progression and also involved in metastasis. The objective of this research was to study the expression and relationship among iNOS, VEGF, COX-2, angiogenesis and their clinico-pathological correlation in OSCC. In this study, standard indirect immunohistochemical technique using polyclonal antibodies specific to human iNOS, VEGF, COX-2 and CD31 was performed in formalin-fixed paraffin-embedded tissue sections of 66 OSCC samples. The staining patterns and intensity are measured and analyzed statistically. The results showed that epithelial components of squamous cell carcinomas demonstrated moderate to intense staining for iNOS, VEGF and COX-2. iNOS shows correlation with cervical lymph node metastasis and tumor staging (TNM) of the patients and angiogenesis. VEGF shows correlation with tumor grading, tumor staging and angiogenesis. COX-2 shows correlation with cervical lymph node metastasis. In conclusion, the expression of iNOS, VEGF and COX-2 exists in OSCC. The data provided show the expression of these chemical mediators associated with carcinogenesis and angiogenesis in OSCC. It can be the primary database before using angiogenesis drug against these mediators for OSCC treatment.

Key words: *Inflammation-induced cancer, iNOS, VEGF, COX-2, angiogenesis, squamous cell carcinoma, oral.*

Introduction

Many evidences show that genetic plays important roles in the cancer risk, however heredity alone cannot explain the etiologies of every cancers. In addition, alteration at the protein level as well as environmental factors can also induce carcinogenesis especially in cancer with known risk factors such as cancer associated with inflammation (1).

Although inflammation has long been known as a localized protective reaction of tissue to irritation, injury, or infection, there has been a new realization about its role in a wide variety of diseases. While acute inflammation is a part of the defense response, chronic inflammation has been found to mediate a wide variety of diseases, including cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases (2). Several pro-inflammatory gene products have been identified that mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Among these gene products, inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), are the current molecules showing close relation to carcinogenesis and angiogenesis.

There are some studies focusing on the roles of iNOS, VEGF and COX-2 in OSCC (3). An analysis of the current literature suggested that iNOS, VEGF and products from COX-2 pathways were involved in the regulation of several biologic processes responsible for tumor growth, such as host immune response, proliferation, resistance to apoptosis and tumor angiogenesis (4). COX-2 activation induced epithelial cells by a variety of stimuli seems to involve in the regulation of tumor angiogenesis by controlling VEGF (5).

Some studies suggested that NO may promote cancer progression by controlling tumor angiogenesis (6). in vivo studies supported that idea and showed interaction between NOS and COX-2 pathways in inflammation (7). In addition, several studies in human carcinoma cell lines have shown that COX-2 activation and VEGF production can be abolished by COX inhibitors, indomethacin and celecoxib. (8).

However, only few data exist regarding their possible correlation with clinico-pathological data in oral squamous cell carcinoma (OSCC). In addition, there are no studies in Thai population on the expression of these proteins in cancer which might be different from Caucasian population in previous studies. This is the main rationale and idea for this study.

Material and Methods

1. Specimens collection

Sixty six OSCC specimens from biopsy or surgical specimen at the Department of Oral Pathology, Faculty of Dentistry, Chulalongkorn University since 1999–2006 were obtained. The specimen included OSCC from various sites such as tongue, buccal mucosa, gingiva, palate, retromolar, lip and others.

Histopathologic slides were prepared from formalin-fixed, paraffin-embedded archival specimens. The tissue sections were cut at 4-µm thick, initially stained with hematoxylin and eosin (H&E) and examined under light microscope by a consultant histopathologist, both to confirm the diagnosis and grading of tumor. The tissue sections and patients' history were reviewed for staging and grading. Other clinical data such as patients age, gender, lymph node status and recurrent were collected. Cases were excluded if the specimen included any other associated pathology (e.g. chronic fungal, bacterial infection or other tumors).

2. Immunohistochemical technique

Paraffin-embedded blocks from the tumor and positive controls were cut at 4-µm thick and placed on lysine coated slides and then processed using standard immunohistochemical technique. Individual OSCC specimen was treated in the same manner but with the omission of the primary antibody also served as internal negative experimental controls.

2.1. Antigen retrieval and immunostaining

For iNOS (polyclonal rabbi anti-human, Santacruz, USA), and VEGF (polyclonal rabbi anti-human, Santacruz, USA), the tissue sections slides were treated with a boiling solution of freshly prepared Tris EDTA buffer, pH 9.0 in microwave oven for 10 min. After cooling down to room temperature, the tissue sections were blocked the nonspecific reaction with normal goat serum at the dilution of 1:100 for 10 min. The sections were incubated in a moist chamber at 4ºC overnight with the primary antibodies.

Each primary antibody specific to iNOS and VEGF was used at the dilution of 1:100. Then, slides were rinsed in Tris-buffered saline twice before being treated with goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody at dilution of 1:100 for 60 min. at room temperature.

For COX-2 staining, polyclonal goat anti-human COX-2 antibody (polyclonal goat anti-human, Santacruz, USA), (1:25) and rabbit anti-goat antibody HRP-conjugated (1:100) were used as primary and secondary antibodies, respectively.

The immunohistochemical reaction was visualized by developing the slides in 3, 3´ diaminobenzidine tetrahydrochloride (Vector Laboratories, USA) and counterstaining with Mayer's hematoxylin. The tissue sections were then dehydrated, cleared and mounted. The experiment was performed in triplicate.

2.2. Immunoreactivity

The sections were evaluated under a Nikon Eclipse 800 microscope (Nikon Corporation, Japan) with a magnification of ×200. Only the cancerous tissues were evalu-

ated. In the sections displaying heterogeneity, the mean average score were used for analysis. Slides were randomly reviewed so as to minimize possible bias. Staining was scored by a consultant histopathologist and the researcher, by evaluating both the percentage of stained cells and the intensity of the stain within 5 representative regions of each specimen. For iNOS and VEGF expression, cytoplasmic staining was scored (9). For COX-2 expression, only plasma membrane of malignant epithelial cells was regarded as COX-2 positive staining (6). The region of staining viewed at a magnification was scored as follows.

The intensity of the stain was on the following scale: 0, no staining seen; 1, mild staining; 2, moderate staining; 3, intense staining.

The area of staining was evaluated as follows: 0, no stained cells in any microscopic field; 1, less than 25% of tumor cells stained positively; 2, between 25 and 50% of tumor cells stained positively; 3, between 50 and 75% of tumor cells stained positively; 4, greater than 75% of tumor cells stained positively.

The sum between area and intensity of staining were used for statistical analysis as described by Brennan et al. (9). For example, if the intensity of stain was graded as 3, the area of staining was graded as 4, the sum is 3 plus 4 which is 7. In this analysis, the minimum score was zero and the maximum was seven.

2.3. Microvessel density analysis

To determine microvessel density (MVD) which represent the cancer angiogenesis, immunostaining with anti-CD31 mouse monoclonal antibody, clone JC31, Dako, Denmark, at dilution 1:100 was used as primary antibody. The procedure was the same as in iNOS and VEGF immunohistochemistry except blood vessels were used as a positive control and EnVision System (Dako, Denmark) was used instead of secondary antibody.

CD31-stained sections were scanned at low magnification (×40) to determine areas with the highest number of microvessels or hot spots, based on the criteria of Weidner et al. (10). Within these hotspots, the microvessel density was counted. Three high power fields were identified on each slide, and the MVD were calculated as the mean number of vessels on a×200 field. It was expressed as the absolute number of microvessels per 0.74 mm2 (×200 fields).

3. Statistical analysis

Expression of iNOS, VEGF and COX-2 in OSCC and the association with clinical and histopathological data were reported and analyzed by Non-parametric Mann-Whitney test for two group differences, Kruskal-Wallis test for three group differences and Spearman Rank correlation for correlation analysis. All statistical analyses were performed with SPSS statistical software package version 15.0 (SPSS, Chicago).

Results

1. Clinical characteristics of OSCC patients

Specimens were taken from 66 patients with an age range of 38 to 90 years (mean ± SD = 64.93 ± 11.93). Forty three patients (65.2 %) were female, the rest 23 (34.8%) were male. The summary for clinical characteristics of the patients is shown in (Table 1).

Table 1. Clinical characteristics of OSCC patients.

	N (%)	
Gender		
Male	23	(34.8)
Female	43	(65.2)
Tumor sites		
Gingiva	30	(45.5)
Tongue	9	(13.6)
Buccal mucosa	7	(10.6)
Floor of mouth	7	(10.6)
Palate	5	(7.6)
Lip	5	(7.6)
Retromolar	2	(3.0)
Periapical region	1	(1.5)
Tumor grade		
Well differentiated	42	(63.6)
Moderately differentiated	15	(22.7)
Poorly differentiated	9	(13.7)
Tumor stage		
Stage I	24	(36.4)
Stage II	8	(12.1)
Stage III	19	(29.1)
Stage IV	15	(22.4)
Lymph node metastais		
Yes	38	(57.6)
No	28	(42.4)
Recurrence		
Yes	13	(19.7)
No	53	(80.3)

* Age range between 38-90 years (mean ± SD = 64.93 ± 11.93)

2. Immunohistochemistry and statistical analysis

Positive controls with parotid tissue, the ducts of which stained intensely for iNOS and VEGF were observed. Inflammatory cells stained served as positive for COX-2 were also observed.

2.1. iNOS expression

iNOS expression was observed in the cytoplasm of squamous cell carcinoma (fig.1). Fifty eight of 66 cases were positive for iNOS with the mean score of 4.94. It was noticed that the inflammatory cells near the tumor interface also shows iNOS staining .

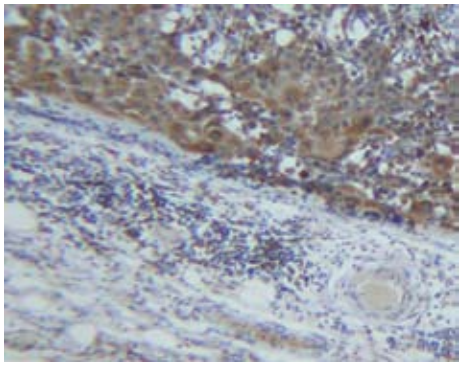

Fig. 1. iNOS expression
iNOS expression in squamous cell carcinoma (×100 original magnification).

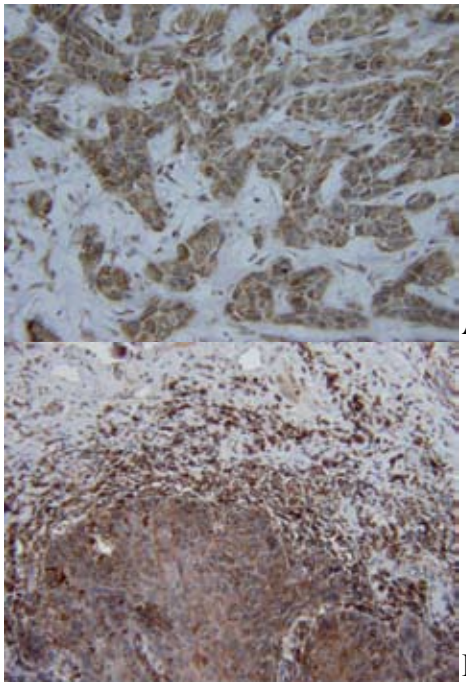

Fig. 2. VEGF expression.
2A: VEGF expression in squamous cell carcinoma.
2B: Positive stain of inflammatory cells near tumor interface of squamous cell carcinoma (x100 original magnification).

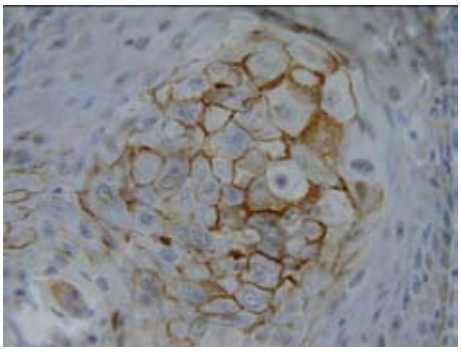

Fig. 3. COX-2 expression
COX-2 expression in squamous cell carcinoma (×100 original magnification).

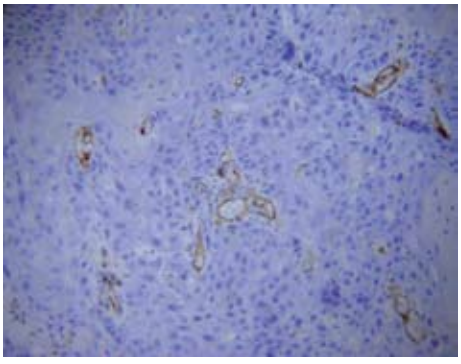

Fig. 4. Microvessels which are positive for CD31 (×200 original magnification).

Table 2. The correlation (rs) between clinico-pathological data of 66 OSCC patients and iNOS, VEGF, COX-2 and MVD.

Clinical data	*rs* (*p*-value)			
	iNOS	VEGF	COX-2	MVD
Tumor grade	0.195 (0.117)	0.338 (< 0.005)*	0.041 (0.743)	0.491 (< 0.005)*
Tumor stage	0.591 (< 0.005)*	0.726 (< 0.005)*	0.200 (0.108)	0.395 (< 0.005)*
Lymph node status	0.415 (< 0.005)*	0.332 (0.008)	0.474 (< 0.005)*	0.090 (0.472)
Recurrence	0.069 (0.583)	0.200 (0.107)	0.134 (0.284)	0.090 (0.185)

* shows significant p-value for Spearman Rank correlation (p < 0.005)

2.2. VEGF expression

The positive stain of VEGF was determined in 59 of 66 cases (Fig.2A). The mean score was 4.47. The inflammatory cells near tumor interface of OSCC were shown to be positive for VEGF as in iNOS staining (fig.2B).

2.3. COX-2 expression

The mean score of COX-2 positivity was 4.29. The positivity in plasma membrane of OSCC was observed in 55 of 66 cases (fig.3).

2.4. MVD Analysis

MVD in tumor samples (fig.4) varied between 11 and 78 (mean, 33.94; SD, 20.01).

2.5. Clinico-pathological correlation analysis

Clinico-pathological correlation analysis was shown in (Table 2). The iNOS expression correlated with lymph node status ($rs=0.42$, $p<0.05$) and tumor staging ($rs=0.59$, $p<0.05$) but not with the tumor grading ($rs=0.20$, $p=0.117$) and tumor recurrent ($rs=0.07$, $p=0.583$). The VEGF expression correlated with tumor grading ($rs=0.34$, $p<0.05$) and tumor staging ($rs=0.73$, $p<0.05$). COX-2 expression correlated with only lymph node status ($rs=0.47$, $p<0.05$). MVD correlated with tumor grading ($rs=0.49$, $p<0.05$) and tumor staging ($rs=0.40$, $p<0.05$)

Discussion

It has been previously suggested that iNOS activity could be regarded as a novel biological marker for assessing tumor progression (11). iNOS expression was reported in many carcinoma (12). Some reported that iNOS expression correlated with lymph node status but not with tumor differentiation (13). In this study, iNOS expression correlated with lymph node status and tumor staging but not tumor grading. All of which has been previously reported by Brennan in UK population (9). The findings are in agreement with previous studies of iNOS expression in breast cancer (14) and iNOS activity in head and neck cancer (15).

VEGF is the only angiogenesis peptide known to act specifically on endothelial cells. It is therefore considered to be the most important factors that promote angiogenesis. The relationship between NO and VEGF has been reported (9,16). Ziche et al. showed that NO was an upstream signal for VEGF-related kinases and also found that breast carcinoma cells that overexpressed VEGF require the NO pathway to induce angiogenesis in vivo (17).

In our study VEGF and MVD shows positive correlation with tumor grading and tumor staging of the patients which are in agreement with previous studies (18). Although some immunohistochemical studies (19) did not find a correlation of VEGF expression in oral dysplasia or carcinoma, this is likely to be a result of differences in the monoclonal antibodies used in recognizing the various isoforms of VEGF.

Although we confirmed the relation and clinico-pathological correlation among iNOS, VEGF and MVD. Further research is required to establish the relationship among these proteins more carefully. Because there are five VEGF isoforms (20), it would be interesting to determine whether one or more of these proteins are involved in this interaction with iNOS and MVD in OSCC. It would also be interesting to compare the results obtained with dysplastic changes that are often found at the margins of invasive oral cancer.

Overexpression of COX-2 may provide several advantages for tumor growth or progression. The expression of COX-2 has been proven to be related with the degree of dysplasia (21). This study shows COX-2 expression in OSCC and we have found that COX-2 correlated with lymph node status but not with tumor grading and staging. Therefore, the expression of COX-2 may be advantageous for assessing the lymph node metastasis in the patients with OSCC.

Overall this study provides evidence for a strong link between chronic inflammation and oral cancer. Thus inflammatory biomarkers as described here can be used to monitor the progression of the disease. These biomarkers can also be exploited to develop new anti-inflammatory drugs to prevent and treat cancer. These drugs can also be used as adjuvant to the currently available chemotherapy and radiotherapy.

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