

## COLOCALIZATION OF VEGF AND INOS IN PARENCHYMAL AND STROMAL CELLS OF ORAL SQUAMOUS CELL CARCINOMA

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### ABSTRACT

**Background and objectives:** Tumor angiogenesis and inflammation perception are intricately linked to cancer progression. Vascular endothelial growth factor (VEGF) controls distinct procedures in angiogenesis of pathological conditions in metabolic functions of cancer cells with the inflammatory cytokine inducible nitric oxide synthase (iNOS) in parenchymal together with stromal cells of oral squamous cell carcinoma (OSCC) as well as assessing microvessel density (MVD).

**Methods:** Immunohistochemistry was applied to analyze VEGF, iNOS as well as CD31 expression within 31 OSCCs paraffin blocks, which grouped into 13 well differentiated, 10 moderately differentiated, and 8 poorly differentiated (paraffin blocks OSCC).

**Results:** Both VEGF and iNOS exhibit strong expression in both parenchymal and stromal cells in all grades of OSCC together with increased MVD.

**Conclusion:** Enhanced expression of VEGF and iNOS together with MVD potentially correlate with OSCC grades.

**KEY WORDS:** VEGF, iNOS, parenchymal, stromal, OSCC

### INTRODUCTION

Although the concept of inflammation as a localized defensive response to trauma has been firmly established for a considerable duration, it assumes a noteworthy function in a wide array of ailments.<sup>1</sup> Moreover, acute inflammation is regarded as an indispensable constituent of the defense mechanism, while chronic inflammation has been

recognized as the agent responsible for multiple diseases, encompassing cancer.<sup>2</sup> Angiogenesis is crucial for tumor growth and survival to provide both purposes nutrients and waste elimination. Moreover, tumors cannot enlarge or metastasize. The process of angiogenesis is maintained through growth factors production from tumor as well as stromal cells.<sup>3,4</sup>

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Several gene products with pro-inflammatory properties have been discovered, which play a key role in inhibiting death and division of cells, construction of novel blood vessels, the spread of cancerous cells, and the colonization of new tissues. Within this group of gene products, iNOS as well as VEGF are presently familiar as molecules that have a strong association with the development of cancer inflammation and angiogenesis respectively.<sup>5,6</sup>

There have been several investigations that have concentrated on examining the functions of iNOS in addition to VEGF in oral squamous cell carcinoma (OSCC) also their contribution in the regulation of various biological processes responsible for the growth of tumors.<sup>7</sup> These processes include the host immune response, cell proliferation, programmed cell death resistance, and the development of new vasculature within tumor environments.<sup>8</sup>

VEGF activation in epithelial cells, brought about by a range of stimuli, appears to contribute to the control of tumor vascularity. A few studies have suggested that nitric oxide (NO) might promote the progression of cancer by exerting control over the formation of tumor blood vessels (new). In addition, iNOS role in the process of tumor development is complex including both pro-tumor and anti-tumor properties. Furthermore, microenvironment release of iNOS and VEGF is strictly connected with tumor poor prognosis.<sup>9,10</sup>

Numerous evidences on the roles played by VEGF and iNOS in the development of cancer leads us to postulate the possibility that the coexistence of VEGF and iNOS could be potentially correlated with different grades of OSCC.

## MATERIAL AND METHODS

### Samples

The current investigation was conducted on 31 paraffin blocks that were diagnosed as oral squamous cell carcinoma, with varying grades of histopathological differentiation (well; 13), (moderately; 10), and (poorly; 8). The blocks were obtained from the archived files of patients at Department of Oral

Pathology, Faculty of Dentistry, University of Tanta. For H&E and immunohistochemical staining, serial sections were prepared by cutting 5  $\mu$ m slices from the paraffin blocks.

The present study plan and events was done following the research rules adopted by the Research Ethics Board, Faculty of Dentistry, Tanta University. Records (paraffin-embedded tissue blocks) were obtained from Tanta University, Oral Pathology Department, Faculty of Dentistry.

### Antibodies

Monoclonal antibody (Mouse) for VEGF (Sc-7269) was obtained from Santa Cruz Biotechnology, Inc. Polyclonal antibody (Rabbit) against iNOS (RM1017) was gotten from Abcam. Mouse monoclonal antibody against CD31 (JC70A, IgG1) Dako (Denmark) supplied.

### Protocol for Immunohistochemistry

Staining for Immunohistochemistry were accomplished with Dako system; ChemMate Envision in consecutive sections, as designated somewhere else.<sup>11</sup> Deparaffinized sections were rehydrated over graded alcohol jars, after that blocking of endogenous peroxidase actions with 0.003% hydrogen peroxide in methanol for at room temperature 30 min, and they were washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4). Slides of iNOS were then placed in the autoclave in buffer of citric acid (pH 6.0) for 10 min at 121°C according to the producer's instruction. CD31 slides were pretreated with 0.2% trypsin (type II; Sigma-Aldrich, USA) in 0.01 M Tris-HCl (pH 7.6) comprising 0.1% CaCl<sub>2</sub> at 37°C for 30 min. Sections were raised with 5% milk protein (Morinaga Milk Industry Co. Ltd, Tokyo, Japan) in 0.01 M PBS encompassing 0.05% Triton X-100 for 60 min at 37°C to block non-specific protein binding locations. Then, incubation with appropriate dilution of primary antibodies, 1:50 for VEGF and 1:200 for iNOS at fridge. After incubations, slides were rinsed with PBS and incubated with the Envision reagents for 1 hour at temperature of the room and treated with 0.02%

3,3'- diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide to reveal the response results. Lastly, slides were counterstained with hematoxylin. For antibodies control, the primary antibodies were substituted with pre-immune IgGs (mouse or rabbit).

**Immunohistochemical Assessment**

After slides were revealed at low power magnification, each case three random fields were chosen at high power magnification by means of objective lens 40x. Characteristic parts for each category were shot on a Nikon Eclipse microscope armed with a DXM1200C digital camera (Nikon, Japan). Cytoplasm immunoreactivity designate positive VEGF and iNOS expression in both OSCC parenchymal and stromal cells. The intensity of immunopositivity and percent of positive cells were evaluated. Numbers of cells with single nucleus that were reveal for VEGF and iNOS were separately totaled manually on serial sections. Results of immunostaining were scored as designated by Bencze J et al.<sup>12</sup> Intensity of color staining was rated as follows: none-0, weak-1, moderate-2 and strong-3.<sup>12</sup>

**Microvessel density (MVD)**

To find the most heavily vascularized areas, three areas were captured (hotspot) at high power magnification using a 20x objective lens. Microvascular

density (MVD) measurement was done manually in a 0.25 x 0.25 mm unit field. Microvascular numbers were defined as CD31positive cells or their collections with or without lumina.<sup>9</sup>

**Analysis of Study Data**

Data were assembled for the present study, arranged and analyzed statistically using the “SPSS 20” (Statistical Package for Social Studies) (SPSS Inc., Chicago, Illinois, USA). The probability value (p-value with 0.05) in significance assessment was utilized.

**RESULTS**

*1) VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (well differentiated)*

In OSCC (well differentiated); where cancerous epithelial cells invading underlying connective tissue with increased formation of keratin pearls (Fig. 1A), VEGF revealed weak expression pattern in the cytoplasm of parenchymal OSCC cells and weak expression pattern in the OSCC stromal cells mainly fibroblasts and endothelial cells of cancer associated blood vessels (Fig. 1B). There was moderate expression of iNOS in peripheral cells of OSCC island and weak expression pattern for stromal cells of OSCC (Fig. 1C). The mean MVD is 11.3 (Table 1).

TABLE (1) VEGF and iNOS expression patterns in OSCC different grades

OSCC grade	Cellular localization	MVD	VEGF expression	iNOS expression
Well differentiated OSCC	Parenchymal	11.3	(+) Weak	(++) Moderate
	Stromal		(+) Weak	(+) Weak
Moderately differentiated OSCC	Parenchymal	15.7	(+++ ) Strong	(+++ ) Strong
	Stromal		(+++ ) Strong	(+++ ) Strong
Poorly differentiated OSCC	Parenchymal	21.1	(+++ ) Strong	(+++ ) Strong
	Stromal		(+++ ) Strong	(+++ ) Strong
			*P-value < 0.05	*P-value < 0.05



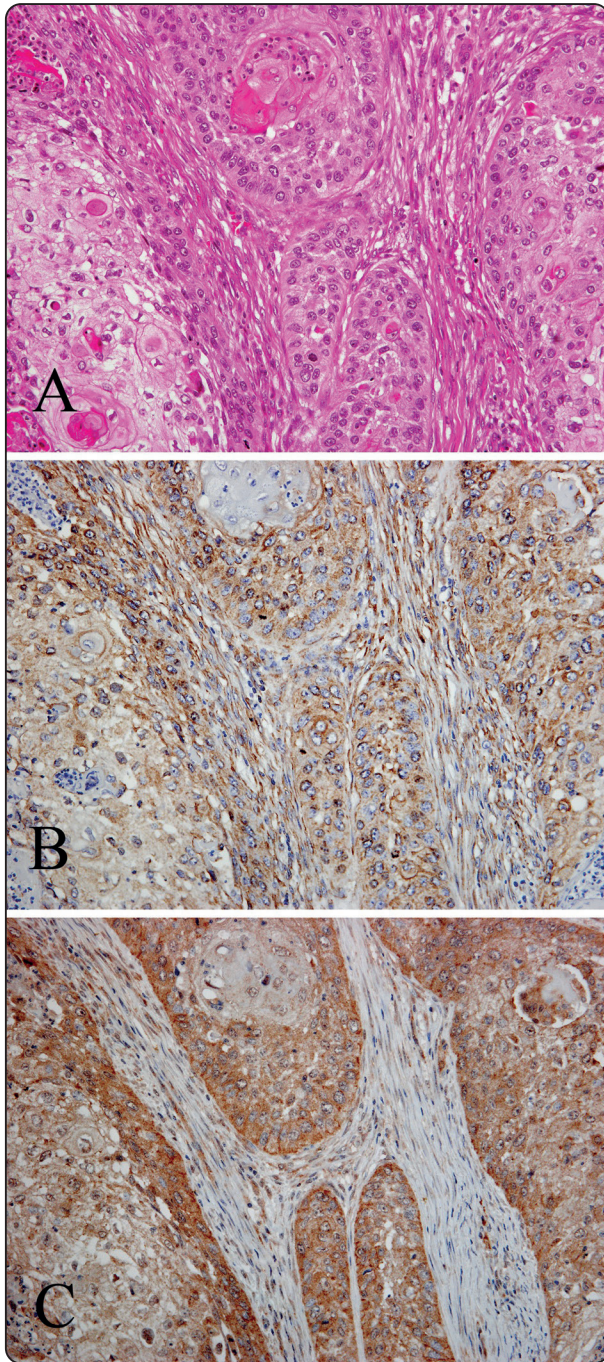


Fig. (1) A photomicrograph of H&E-stained tissue sections reveal VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (well differentiated). In well differentiated OSCC; cancerous epithelial cells invade the adjoining connective tissue with increased keratin pearl formation (A), VEGF revealed weak expression pattern in the cytoplasm of OSCC cells and weak expression pattern in the OSCC stromal cells mainly fibroblasts and endothelial cell of blood vessels (B). There was moderate expression of iNOS in peripheral cells of OSCC island and weak expression pattern for stromal cells (C). Hematoxylin and eosin (HE) (A) and immunoperoxidase stains for VEGF (B) and iNOS (C); (A -C)  $\times 100$ .

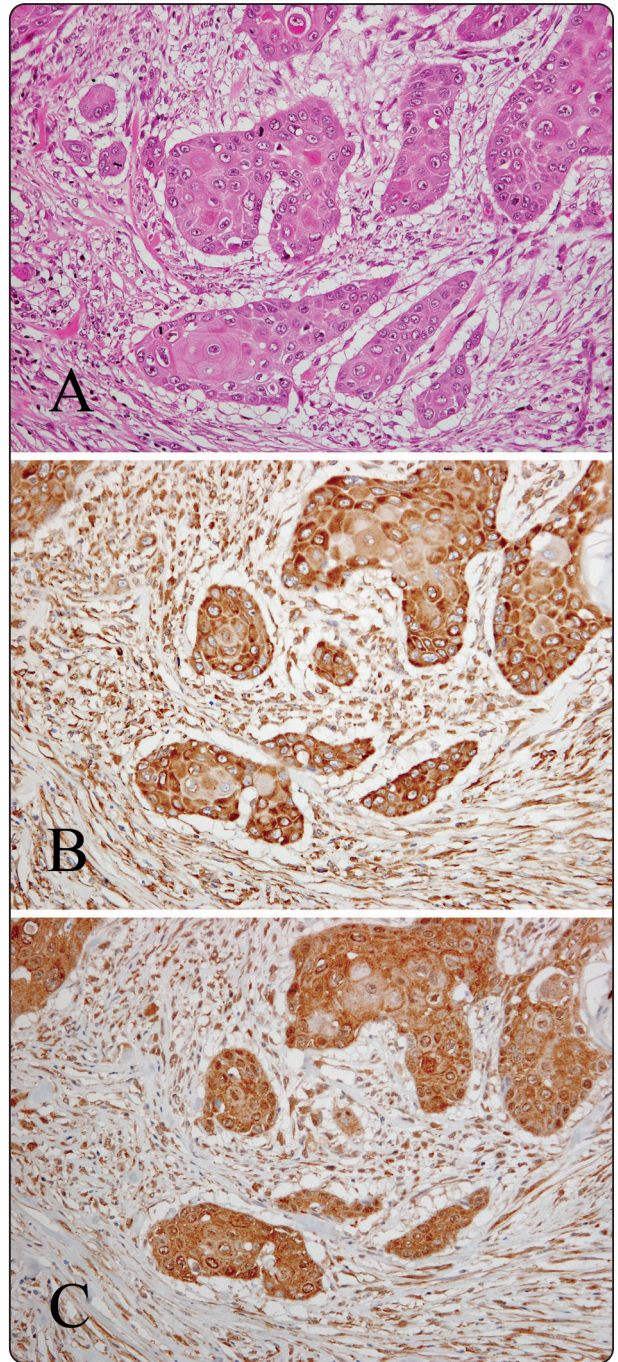


Fig. (2) A photomicrograph of H&E-stained tissue sections reveal VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (moderately differentiated). In moderately differentiated OSCC; where cancerous epithelial cells invading adjoining connective tissue with less keratin pearl formation (A), VEGF revealed strong expression pattern in the cytoplasm of OSCC cells and stromal cells mainly fibroblasts and endothelial cell of blood vessels (B). There was strong expression of iNOS in both parenchymal and stromal cells of OSCC (C). Hematoxylin and eosin (HE) (A) and immunoperoxidase stains for VEGF (B) and iNOS (C); (A -C)  $\times 100$ .



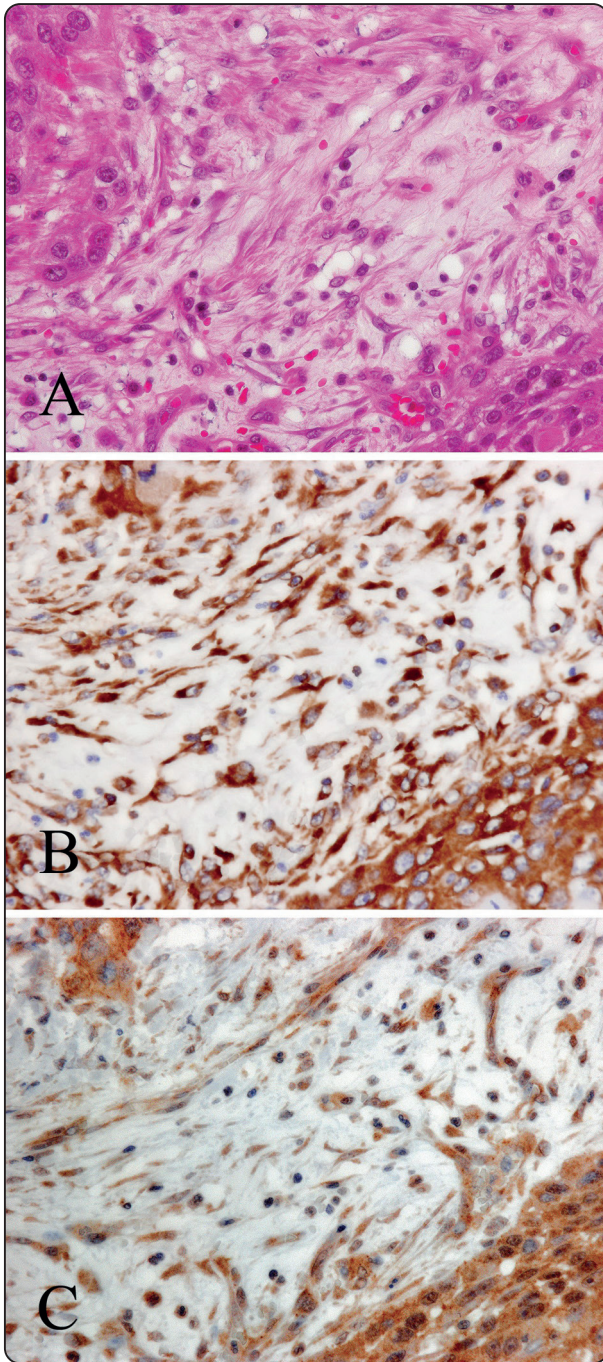


Fig. (3) A photomicrograph of H&E-stained tissue sections reveal VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (poorly differentiated). In poorly differentiated OSCC; where cancerous epithelial cells invading adjoining connective tissue with streaming pattern (A), VEGF revealed strong expression pattern in the cytoplasm of OSCC cells and stromal cells mainly fibroblasts and endothelial cell of blood vessels (B). There was strong expression of iNOS in both parenchymal and stromal cells (C). Hematoxylin and eosin (HE) (A) and immunoperoxidase stains for VEGF (B) and iNOS (C); (A-C)  $\times 100$ .

### 2) *VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (moderately differentiated)*

In OSCC (moderately differentiated); where cancerous epithelial cells invading underlying connective tissue with less keratin pearl formation (Fig. 2A), VEGF revealed strong expression pattern in parenchymal OSCC cells and stromal cells cytoplasm (Fig. 2B). There was strong expression of iNOS in both parenchymal and stromal cells of OSCC (Fig. 2C). The mean MVD is 15.7 (Table 1).

### 3) *VEGF and iNOS expression in parenchymal and stromal cells of oral squamous cell carcinoma (poorly differentiated)*

In OSCC (poorly differentiated); where malignant epithelial cells invading underlying connective tissue with streaming pattern (Fig. 3A), VEGF revealed strong expression pattern in parenchymal OSCC cells and stromal cells cytoplasm (Fig. 3B). There was strong expression of iNOS in both parenchymal and stromal cells of OSCC (Fig. 3C). The mean MVD is 21.1 (Table 1).

Both VEGF and iNOS expression in parenchymal and stromal OSCC cells were highly significant between all grades combined with increased MVD; P-value  $\leq 0.05$  (Table 1).

## DISCUSSION

Angiogenesis is a crucial step for a tumor's effective growth, invasion, and metastasis. VEGF is widely acknowledged to be the greatest influential agent for tumor angiogenesis. Although VEGF and iNOS have been linked to different malignancies, less is identified around how these two proteins interrelate and express in OSCC. Herein, the association between tumor grades and VEGF and iNOS immunohistochemistry in OSCC was inspected. Our findings demonstrated a substantial link between the VEGF and iNOS expression and the tumor pathological OSCC grades harmoniously with increased microvessel density (MVD) (P $\leq 0.05$ ).

VEGF and iNOS have been proposed as biological indicators for monitoring the development of tumors. The only angiogenesis protein that has been shown to target endothelial cells selectively is VEGF. As a result, it is thought to be the primary element that promotes angiogenesis. It has been observed that the link between NO and VEGF is such that NO functions as an upstream signal for VEGF-related kinases. Additionally, it has been found that *in vivo* angiogenesis is induced in cancer cells that overexpress VEGF by the NO route.<sup>13</sup>

Overall, this study shows a clear correlation between oral cancer and chronic inflammation, with iNOS as a potent inflammatory biomarker being able to track the disease's advancement. This biomarker may also be utilized to create novel anti-inflammatory medications that prevent cancer and utilized as a supplement to the radiation therapy and chemotherapy.<sup>14, 15</sup>

In this study VEGF shows positive correlation with tumor grading. Even though some immunohistochemistry work did not find a association expression of VEGF in epithelial dysplasia or cancer of oral cavity, due to differences in antibodies used in identifying the several isoforms of VEGF.<sup>6, 16</sup>

It has been proposed that iNOS action could be observed in different cancer types as tumor progression biological indicator. Some informed that iNOS expression was consistent with lymph node involvement but not tumor differentiation grade. Herein in our study, iNOS expression associated with tumor histological grade.<sup>17, 18</sup>

Induction of iNOS results in nitric oxide release and increase in permeability of tumor blood vessels as well as hasten nutrient supply of tumor cells and subsequent growth of tumor.<sup>1</sup> The aforementioned information suggests a complex relationship between VEGF and iNOS during the tumor development process. As OSCC tumor tissues advance, there is a corresponding increase in VEGF expression and iNOS expression in consistent with increased MVD from well to poorly differentiated OSCC.<sup>4, 19, 20</sup>

In conclusion, we have demonstrated strong relation between OSCC pathologic grade with intensity of expression of VEGF and iNOS together with increased MVD indicating that VEGF and iNOS are related to OSCC angiogenesis. This relation might provide more credence to the idea that blocking VEGF and/or iNOS can be used as OSCC therapeutic approach.

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