Correlation of hypoxia-inducible factor-1 alpha (HIF-1α) and vascular endothelial growth factor (VEGF) expressions with clinico-pathological features of oral squamous cell carcinoma (OSCC)

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Abstract

Oral squamous cell carcinoma (OSCC) is a characteristic aggressive tumor representing a significant public health threat all over the world. Up to 50–60% of solid tumors may exhibit hypoxic tissue areas that are heterogeneously distributed within the tumor stroma. Hypoxia inducible factor-1α (HIF-1α) is a central regulator in the adaptive cellular response to hypoxia; however its role is still uncovered. Vascular endothelial growth factor (VEGF) is an important molecule playing crucial role not only in inflammation, but also in angiogenesis and thus tumor growth and progression. So, the aim of this work is to investigate the expression of HIF-1α and VEGF in OSCC and correlate their expressions with clinical and histopathological features of OSCC. In this work, tissue specimens from a total of 45 cases with OSCC were stained immunohistochemically with HIF-1α and VEGF antibodies and examined microscopically.

The results of this study revealed that HIF-1α and VEGF expressions appeared to be significantly positive and directly related to histopathological grades, lymph node (LN) status as well as clinical stages of OSCC. Moreover, HIF-1α expression was significantly correlated to angiogenic activity measured by VEGF immunostaining. Thus, it was concluded that HIF-1α and VEGF expressions were up-regulated with increased malignancy and can be used as predictive markers of tumor behavior.

Keywords: OSCC; HIF-1α; VEGF

1. Introduction

Worldwide, oral cancer (OC) is a public health problem, representing the sixth most common malignant neoplasm. Oral squamous cell carcinoma (OSCC) is considered the most frequent of all oral neoplasms representing about 95% of these neoplasms [1].
The process of tumorigenesis involves numerous changes in tumor microenvironment (TME) that lead to the production of new components and/or upregulation of its molecules that play significant roles in tumor progression. These changes that occur in OSCC progressively increase the ability of transformed cells to proliferate and invade. The heterogeneity of these changes explains why tumors at the same clinical stage and localization often show significant differences in their clinical outcomes and treatment responses [2,3].

Hypoxia is one of the most common changes that occur in the TME and it is a common characteristic of human solid tumors, and it can drive malignant cells to undergo adaptive changes that enable them to survive in oxygen-depleted regions. Hypoxia inducible factor-1 (HIF-1) is a transcription factor composed of two subunits namely HIF-1α and HIF-1β. HIF-1β is a constitutively nuclear-located subunit that is found under all oxygen conditions. However, HIF-1α is a highly regulated subunit that is extremely labile in normoxia with a half-life of less than 5 min. The rapid and continuous degradation of HIF-1α is effectively blocked if oxygen availability is reduced. This permits dimerization with HIF-1β to form the active HIF-1 complex [4,5].

HIF-1α contains a unique oxygen-dependent degradation domain (ODDD) that is central to the oxygen-regulated stability of this protein. Specific degradation of HIF-1α in normoxia is triggered through this domain. Therefore, hypoxia increases HIF-1 levels in cells by strongly stabilizing HIF-1α through the inhibition of its rapid degradation by the proteasome [5]. These striking criteria in oxygen sensitivity & trans-activation ability of HIF-1α make it the main functional protein of HIF-1 complex [4]. More than 60 genes have been found to be induced by HIF-1α that are implicated in many different cellular functions such as cell survival, cell proliferation, apoptosis, glucose metabolism and angiogenesis [6].

Many studies have shown that the immunohistochemical (IHC) expression of HIF-1α is associated with a poor prognosis, however others have only shown a trend for poor prognosis and some have not been statistically significant. In three studies, in head and neck (HN) cancer, OC and non-small cell lung cancer, HIF-1α expression predicted a good prognosis [7–9].

One of the studies where HIF-1α expression was a good prognostic factor showed a strong correlation between HIF-1α and the expression of pro-apoptotic factors such as caspase-3, Fas, and Fas ligand [9].

In addition to prognosis, HIF-1α expression was also investigated in some studies on esophageal cancer patients to predict response to chemo-radiotherapy. HIF-1α expression predicted a poor treatment response [5,10].

Liang et al. [11], has reported that HIF-1α over-expression was correlated significantly with the clinical stage, histopathological grade, lymph node (LN) metastasis and worse prognosis in patients with OSCC of the tongue. Furthermore, it was found that HIF-1α over-expression in combination with deficiency or mutation of tumor suppressor genes such as von Hippel Lindu (vHL), p53, and amplification of oncogenes as Akt and Ras was frequently seen in human cancer and these genetic alterations have been associated with tumor growth, invasion and metastasis. Most cancers over-expressing HIF-1α are associated with increased mortality [12].

Angiogenesis is also essential for the development of solid tumors and the survival of their cells. Tumor cells that are located more than 100 mm away from blood vessels become hypoxic. If new blood vessels do not form, tumor clones will be confined within 1–1.5 mm³ diameter. Such clones remain dormant from months to years before they switch to an angiogenic phenotype [13]. In the beginning, angiogenesis develops by incorporating existing host blood vessels; no solid tumors can probably grow more than 2 mm³ unless they synthesize their own network of new microvessels. This angiogenic switch is dependent mainly on hypoxia and oncogenic transformation which occurs in tumor cells, or in an autocrine growth factor loop [14].

Vascular endothelial growth factor (VEGF) is considered a potent angiogenic activator and its associate receptor (VEGFR-2). VEGF is a member of platelet-derived growth factor (PDGF) superfamily of growth factors that triggers mobility and maturation of endothelial cells (ECs) towards the hypoxic environment that allows blood and oxygen to reach hypoxic cells [15]. It has long been established that ECs of tumor-associated neovasculature proliferate 20–2000 times more rapidly than ECs of normal tissue [16].

Macluskey et al. [17] and Carlile et al. [18], observed a significant increase in vascularity during the transition from normal oral mucosa, through different degrees of dysplasia, to invasive carcinoma. Several studies have also reported a close association between tumor angiogenesis and tumor progression from early to late OSCC [19,20]. However, conflicting results were reported relating to HIF-1α and VEGF expressions in HN cancers.

So, the aim of the present work is to investigate the expression and the distribution patterns of HIF-1α and
VEGF immunostainings in OSCC and to correlate their expressions with the clinico-pathological features of OSCC to clarify their role in the biological behavior of this tumor.

2. Material and methods

- The present study included 45 cases with OSCC who were diagnosed and subjected to surgery at Tanta Cancer Center and Oral and Maxillofacial Surgery Department, Faculty of Dentistry, Tanta University. Neither pre-operative chemotherapy nor radiotherapy was performed. Informed consent was obtained from all the patients examined in this study. Detailed case history and thorough clinical examination with clinical staging was performed for each case.

- The fresh tissue specimens of OSCC were fixed in 10% neutral buffered formalin and routinely processed for hematoxylin and eosin (H&E), examined under light microscope and graded histologically.

- For IHC study, the Avidin–Biotin Complex (ABC) technique was used to detect the following reagents:
  - Primary antibodies against HIF-1α (Clone: 54/HIF-1α, Mouse monoclonal, Becton Dickinson (BD) Transduction Laboratories, Franklin Lakes, USA) and VEGF (Clone: SP6, Rabbit monoclonal, Neomarkers, Fremont CA, USA). The Avidin Biotin Complex (ABC) Universal Kit (Neomarkers, Fremont CA, USA) was used.

- The immunostaining procedure was performed according to the manufacturer's instructions. The deparaffinized sections associated with immunostaining kit were processed acting as positive controls whereas negative controls consisting of tissue sections on which primary antibody was replaced with non-immune serum was also performed.

- The sections were evaluated for HIF-1α and VEGF expressions by assessing the site of staining (nuclear and/or cytoplasmic). Scoring the IHC positivity was done using two parameters; staining intensity and percentage of stained positive cells within 4 different microscopic fields of the same section at X200 magnification as follows: Percentage of positive cells: (0) if the tumor cells showed no reactivity of antigen (1), (Focal) if <30% of the tumor cells are positive (2), (Partial) if 30–60% of the tumor cells are positive (3), (Diffuse) if >60% of the tumor cells are positive. The intensity: 1 = [weak], 2 = [moderate], 3 = [strong].

- Final score for each case was the sum of both intensity and percentage and was represented as follows: Negative = (0), Weak = (2–3), Moderate = (4), Strong = (5–6).

- All IHC evaluation was performed by two independent observers blind to clinical information of the cases and the average of both observers’ scoring was used.

- Statistical analysis was done using Monte Carlo exact test by SPSS program V.19 and Spearman's rank correlation coefficient (rho). The p value for significance was adopted at P < 0.05.

3. Results

3.1. Clinical findings

- All the observed clinical findings of OSCC cases are showed in Table 1. It was found that the mean age of the studied cases was 54 years old and the tongue was the most commonly affected site (17 cases) followed by buccal mucosa (10 cases). Twenty cases were classified clinically as advanced stages (Stage III and IV) and 22 cases of OSCC appeared as exophytic fungating masses with or without surface ulcerations (Fig. 1A), while 17 cases appeared as endophytic ulcerative lesions (Fig. 1B). The remaining 6 cases appeared clinically as white and/or red lesions (Fig. 1C). Thirteen cases showed LN metastasis and 2 cases recurred locally.

Table 1

| Clinical data                      | Range | Mean ± SD |
|-----------------------------------|-------|-----------|
| Age (years)                       | 28–76 | 54.5 ± 11.18 |
| Sex                               |       |           |
| Male                              | 24    | (53.3)    |
| Female                            | 21    | (46.7)    |
| Size of primary tumor             |       |           |
| <2 cm                             | 13    | (28.8)    |
| 2–4 cm                            | 16    | (35.6)    |
| >4 cm                             | 16    | (35.6)    |
| Site of primary tumor             |       |           |
| Tongue                            | 17    | (37.8)    |
| Buccal mucosa                     | 10    | (22.2)    |
| Gingiva                           | 5     | (11.1)    |
| Lip                               | 4     | (8.9)     |
| Palate                            | 4     | (8.9)     |
| Floor of the mouth                | 3     | (6.7)     |
| Alveolar mucosa                   | 2     | (4.4)     |
| Lymph node involvement            | 13    | (28.9)    |
| Clinical stage (UICC)             |       |           |
| Stage I                           | 13    | (28.9)    |
| Stage II                          | 12    | (26.7)    |
| Stage III                         | 12    | (26.7)    |
| Stage IV                          | 8     | (17.7)    |
| Clinical presentation             |       |           |
| Exophytic                         | 22    | (48.9)    |
| Endophytic                        | 17    | (37.8)    |
| Red and/or white lesion           | 6     | (13.3)    |
| Duration                          |       |           |
| <12 months                        | 20    | (44.4)    |
| >12 months                        | 25    | (55.6)    |
| Local recurrence                  | 2     | (4.4)     |
| Total                             | 45    | (100)     |
3.2. Conventional hematoxylin and eosin staining (H&E)

- By light microscopic examination, 19 cases out of the 45 cases of OSCC were diagnosed as well-differentiated (Grade I) (Fig. 5a), 17 cases were diagnosed as moderately differentiated (Grade II) (Fig. 6a) and the remaining 9 cases were poorly differentiated tumors (Grade III) (Fig. 7a).
- It was observed that 14 out of 25 cases with early stages of OSCC (Stage I and II) were well-differentiated histologically and only 3 cases were poorly differentiated tumors. Five cases of OSCC out of 20 with advanced clinical stages (Stage III and IV) were diagnosed as well-differentiated tumors, while 6 cases were poorly differentiated (Table 2) (Fig. 2). The clinical stages showed non-significant correlation to the histopathological grades (p = 0.067, rho = 0.276) (i.e. the lesser degree of differentiation of the tumor cells is not essentially associated with more advanced clinical stage of OSCC).

3.3. Immunohistochemical staining

3.3.1. Correlation of antigen reactivity to the clinical findings

- Regarding the age of the patients, most cases of OSCC at the 7th and 8th decades of life showed high (moderates and strong) HIF-1α and VEGF reactivities representing 68.7% (11 out of 16 cases) and 81% (13 cases) for each antigen respectively. However, all patients at the 3rd and 4th decades of life (4 cases) showed weak expressions of HIF-1α and VEGF except one case of them revealed no
positivity of HIF-1α. OSCC cases from the tongue showed the highest levels of HIF-1α and VEGF stainings representing 67% and 35% respectively. However, the correlation was not significant between the site of OSCC and expressions of the studied antigens (Tables 3 and 4).

- HIF-1α immunostaining increased significantly with advanced clinical stages of OSCC (p = 0.002), hence 11 out of 20 cases with stage III and IV showed strong HIF-1α reactivity, while only 1 case out of 25 with stages I and II showed marked upregulation of HIF-1α expression (Table 5) (Fig. 3). Similarly, VEGF reactivity was significantly correlated to clinical stages of OSCC (p = 0.002), since VEGF was weak in 11 cases of stage I cases. However, 6 cases of stage IV-OSCC showed strong VEGF reactivity and 2 cases were moderately-stained with VEGF, while no cases showed weak VEGF (Table 5) (Fig. 4).

- HIF-1α and VEGF expressions were significantly correlated to LN involvement (p = 0.001 for each) as 9 cases out of 13 with LN metastasis (69%) showed strong staining of both antigens, while 65% of cases (21 out of 32 cases) without any nodal involvement showed negative or weak HIF-1α and VEGF immunostainings. Strong HIF-1α expression was also observed in cases that showed local recurrence.
3.3.2. Correlation of antigen reactivity to the histopathological findings

3.3.2.1. Well-differentiated OSCC (grade I, 19 cases)

- HIF-1α immunoreactivity appeared as a localized or focal granular cytoplasmic and/or nuclear brown staining of mainly the peripheral malignant cell layers in the keratin and epithelial pearls. It was more pronounced around areas of necrosis and keratinization (Fig. 5b and c). In most cases, HIF-1α expression appeared more intense in areas away from the closest blood vessels.

- Cytoplasmic HIF-1α was predominant than nuclear staining or may appear the only expression pattern in 12 cases of well-differentiated OSCC (63%) (Fig. 6b). Almost all of these cases (11 cases) were clinically classified as stage I and II. HIF-1α staining was not positive in only one case (Fig. 5d). HIF-1α expression was also seen in vascular ECs of the tumor stroma in 9 cases (47%) (Fig. 5e).

- VEGF expression appeared mostly as localized cytoplasmic brown staining of the tumor cells with higher intensity at the outer cell layers and less intense toward the center. In addition, stromal cells including inflammatory cells, ECs, fibroblasts also demonstrated cytoplasmic VEGF reactivity especially those cells at the tumor—stroma interface (Fig. 5f). Generally, it was observed that 12 cases of well-differentiated tumors showed weak immunostaining of HIF-1α and VEGF. Only 2 cases revealed strong reactivity of the studied antigens and these cases were classified clinically as stage III and IV with LN metastasis (Figs. 8 and 9) (Table 5).

3.3.2.2. Moderately-differentiated OSCC (grade II, 17 cases)

- Few cases showed localized HIF-1α immunoreactivity either nuclear or cytoplasmic (Fig. 6b), while most cases of moderately-differentiated OSCC demonstrated diffuse HIF-1α expression throughout the entire tumor which was independent of vessel proximity. Nuclear HIF-1α was predominant than cytoplasmic staining in 14 cases (82.3%) (Fig. 6c). Almost all of these cases (16 cases) were clinically
classified as stage III and IV. Endothelial HIF-1α immunostaining was also seen in 12 cases (70%) (Fig. 6g). VEGF reactivity appeared mostly as diffuse moderate brown cytoplasmic staining in the malignant cells with higher intensity at the outer cell layers (Fig. 6d and f).

- By analyzing the expression level of HIF-1α in moderately-differentiated OSCC, it was found that 10 cases demonstrated high scores of immunoreactivity (5 cases were moderate and 5 cases were strong) (Figs. 6c and 8), while 6 cases showed weak HIF-1α immunoreactivity (Figs. 6e and 8). Only one case was not positive for HIF-1α expression. On the other hand, VEGF expression showed high scores of staining in 12 cases (6 were moderate and 6 cases were strong) (Fig. 6d). The
remaining 5 cases revealed weak reactivity for VEGF (Figs. 6f and 9) (Table 5).
- VEGF immunostaining was also observed markedly in the stromal fibroblasts, inflammatory cells and vascular ECs (Fig. 6h).

3.3.2.3. Poorly-differentiated OSCC (grade III, 9 cases)

- HIF-1α expression in most cases of poorly-differentiated OSCC (7 out of 9 cases) appeared mainly as intense diffuse nuclear reaction of the invading malignant cells, with or without cytoplasmic localization (Fig. 7b). Only one case showed cytoplasmic HIF-1α reactivity without nuclear pattern of expression.
- As shown in Table 5, the majority of poorly-differentiated specimens (7 cases) showed high HIF-1α and VEGF expressions (moderate and strong stainings) (Fig. 7b and c). Whereas, only few cases showed weak expression levels of these antigens (2 cases out of 9 for both HIF-1α and VEGF) (Figs. 8 and 9). Interestingly, the cases that demonstrated weak expression of the studied antigen were classified clinically as stage I and II.

### Table 2

| Clinical stages | Histopathological grades | Total |
|-----------------|--------------------------|-------|
|                 | Well differentiated       |       |
|                 | n (%)                    |       |
| Stage I         | 6 (46)                   | 13 (28.9) |
| Stage II        | 8 (66.7)                 | 12 (26.7) |
| Stage III       | 4 (33.3)                 | 12 (26.7) |
| Stage IV        | 1 (12.5)                 | 8 (17.7) |
| Total           | 19 (42.2)                | 45 (100) |
|                 | Moderately differentiated |       |
|                 | n (%)                    |       |
| Stage I         | 5 (38.7)                 | 13 (28.9) |
| Stage II        | 3 (25)                   | 12 (26.7) |
| Stage III       | 5 (41.7)                 | 12 (26.7) |
| Stage IV        | 4 (50)                   | 8 (17.7) |
| Total           | 17 (37.8)                | 45 (100) |
|                 | Poorly differentiated     |       |
|                 | n (%)                    |       |
| Stage I         | 2 (15.3)                 | 13 (28.9) |
| Stage II        | 1 (8.3)                  | 12 (26.7) |
| Stage III       | 3 (25)                   | 12 (26.7) |
| Stage IV        | 3 (37.5)                 | 8 (17.7) |
| Total           | 9 (20)                   | 45 (100) |
Strong VEGF reactivity was also observed in the stromal cells especially fibroblasts at the deep invasive front (Fig. 7f). Generally, in all the studied 45 cases of OSCC HIF-1α and VEGF expressions showed significant correlation with the degree of differentiation of OSCC ($p = 0.001$ for each antigen) (Table 6). Moreover, HIF-1α expression was significantly correlated to tumor cell angiogenesis measured by VEGF reactivity ($p = 0.001$) (Table 7).

4. Discussion

OSCC remains one of the most difficult malignancies to control and there has been little improvement in the survival rate over the past 20 years. Despite many efforts, precise assessment of progression and behavior of OSCC with a view to predict prognosis remains an elusive goal.

This study showed that men were more affected than women; however, the male: female ratio was less pronounced (1.2:1). Similar trends have been reported in literature regarding the gender with increasing incidence in women that has been attributed to increased participation of women in risk factor behaviors such as smoking and alcohol consumption in recent decades[21,22]. In this study, Tongue was the most affected site (37.8%) and mostly associated with metastasis (53.8%). This is supported by previous studies that showed the same results[23–25] and may be explained by the rich blood supply and lymphatic drainage of the tongue.

The present study revealed a non-significant direct correlation between clinical stages and the degree of differentiation of OSCC. These results are consistent

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### Table 3
Expression of HIF-1α in relation to the age and site of OSCC cases.

| Age range (years) | HIF-1α expression | Total |
|-------------------|--------------------|-------|
|                   | Negative | Weak | Moderate | Strong | n (%) |
| 20–39             | 1 (25)   | 3 (75) | 4 | 4 |
| 40–59             | 12 (48)  | 8 (32) | 5 (20) | 25 |
| 60–69             | 1 (8.3)  | 4 (33.3) | 3 (33) | 12 |
| 70–76             | 4 (100)  | 4 |

| Site of OSCC | Tongue | Buccal mucosa | Gingiva and Alveolar mucosa | Lip | Palate | Floor of the mouth |
|--------------|--------|---------------|-----------------------------|-----|--------|-------------------|
| n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| 1 (5.9) | 6 (35.3) | 3 (17.6) | 7 (41.2) | 17 |
| 1 (14.3) | 2 (28.6) | 4 (57.1) | 7 |

| Site of OSCC | Total |
|--------------|-------|
| n (%) | n (%) |
| 6 (35.3) | 6 (35.3) | 5 (29.4) | 17 |
| 4 (40) | 4 (40) | 2 (20) | 10 |
| 3 (42.9) | 3 (42.9) | 1 (14.3) | 7 |

### Table 4
VEGF expression in relation to the age and site of OSCC cases.

| Age range (years) | VEGF expression | Total |
|-------------------|-----------------|-------|
|                   | Weak | Moderate | Strong | n (%) |
| 30–39             | 4 (100) | – | – | 4 |
| 40–59             | 12 (48) | 7 (28) | 6 (24) | 25 |
| 60–69             | 3 (23) | 7 (53.8) | 2 (15.2) | 13 |
| 70–74             | 2 (50) | 1 (25) | 1 (25) | 4 |

| Site of OSCC | Tongue | Buccal mucosa | Gingiva and Alveolar mucosa | Lip | Palate | Floor of the mouth |
|--------------|--------|---------------|-----------------------------|-----|--------|-------------------|
| n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| 6 (35.3) | 6 (35.3) | 5 (29.4) | 17 |
| 4 (40) | 4 (40) | 2 (20) | 10 |
| 3 (42.9) | 3 (42.9) | 1 (14.3) | 7 |

| Site of OSCC | Total |
|--------------|-------|
| n (%) | n (%) |
| 2 (50) | 2 (50) | – | – | 4 |
| 2 (50) | 1 (25) | 1 (25) | 4 |
| 1 (33.3) | 1 (33.3) | 1 (33.3) | 3 |

### Table 5
HIF-1α and VEGF expressions in clinical stages and histo-pathological grades of OSCC.

| Antigen expression | Clinical stages | Histopathological grades |
|--------------------|-----------------|--------------------------|
|                    | Stage I | Stage II | Stage III | Stage IV | Grade I | Grade II | Grade III |
|                    | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) |
| HIF-1α | Negative | 1 | 7 | 1 | 8 | – | – | – | –|
|         | Weak | 10 | 8 | 6 | 50 | 4 | 33 | – | – |
|         | Moderate | 2 | 15 | 4 | 34 | 4 | 33 | 1 | 12 |
|         | Strong | – | – | 1 | 8 | 4 | 34 | 7 | 88 |
| VEGF | Negative | – | – | – | – | – | – | – | – |
|         | Weak | 11 | 85 | 5 | 42 | 3 | 25 | – | – |
|         | Moderate | 2 | 15 | 7 | 58 | 4 | 33 | 2 | 25 |
|         | Strong | – | – | – | 5 | 42 | 6 | 75 | 2 | 11 |

(4.7d–e). Strong VEGF reactivity was also observed in the stromal cells especially fibroblasts at the deep invasive front (Fig. 7f). Generally, in all the studied 45 cases of OSCC HIF-1α and VEGF expressions showed significant correlation with the degree of differentiation of OSCC ($p = 0.001$ for each antigen) (Table 6). Moreover, HIF-1α expression was significantly correlated to tumor cell angiogenesis measured by VEGF reactivity ($p = 0.001$) (Table 7).
with the findings of Dantas et al. [26], who reported the same results in OSCC of the tongue with the tumor size fails to signify any information related to the actual histological condition of the tumor.

Hypoxia is a common characteristic of human solid tumors, and it can drive malignant cells to undergo adaptive changes that enable them to survive in oxygen-depleted regions. It has been suggested that tumor hypoxia plays an essential role in promoting chromosome instability, cancer cell invasiveness, and metastasis. It is associated with aggressive tumor growth and treatment failure in several human solid tumors [2,3].

When correlating HIF-1α reactivity with clinical features of the studied cases regarding the age of the patients, the correlation was positive. This may be due to mutations that are more common to occur in older people. Consequently, these mutations might activate oncogenes that encourage the expression of many transcriptional factors such as HIF-1α and their activation [27].

Angiogenesis is another adaptive response of tumor cells to hypoxia. VEGF is a multifunctional cytokine expressed by both tumor and stromal cells. Its angiogenic effect is based on its ability to induce various

![Fig. 8. Analysis of HIF-1α expression in different histopathological grades of OSCC.](image1)

![Fig. 9. Analysis of VEGF expression in different histopathological grades of OSCC.](image2)

| Variables          | Histopathological grade | rho  | p     |
|--------------------|------------------------|------|-------|
| HIF-1α expression  | 0.572                  | 0.001*|
| VEGF expression    | 0.528                  | 0.001*|

Monte Carlo Exact Test: p = 0.001 (*Significant).

| Variables          | HIF-1α expression | rho  | p     |
|--------------------|-------------------|------|-------|
| VEGF expression    | 0.859             | 0.001*|

Monte Carlo Exact Test: p = 0.001 (*Significant).
responses by ECs during vascular development, including cell proliferation, migration, specialization and survival [28].

In the current study, enhanced VEGF expression also appeared in old-aged patients than in young patients. This could be due to the epigenetic changes that are increasingly recognized as a part of aging as hypermethylation of p53 genes. This may lead to gene silencing and suppress its function, thus facilitate VEGF expression to escape the control of p53 suppressor gene [27,29].

Furthermore, the observed significant correlation between HIF-1α, clinical stages and LN status of OSCC in this study was consistent with that reported by Eckert et al. [30] and Tilakaratne and Nissanka-Jayasuriya. [31], who found a positive correlation between HIF-1α, tumor size, recurrences, nodal and distant metastasis. This may be a result of upregulation of matrix metalloproteinases (MMPs) in hypoxic OSCC that are responsible for degradation of the extracellular matrix (ECM). These findings are indicative that HIF-1α overexpression in OSCC might be responsible for enhancement of tumor invasion as well as metastasis, resulting in poor prognosis of OSCC [32]. Furthermore, Ryu et al. [33], postulated that HIF-1α accumulation in tumor cells modulates α2-integrin and fibronectin (invasion-related factors) favoring invasion by enhancement of new cell-ECM attachments by fibronectin-rich ECM deposition.

Imai et al. [34], also observed downregulation of E-cadherin expression in OSCC cells under hypoxic conditions that leads to loss of cellular cohesiveness, consequently resulting in more invasive and metastatic potential of tumor cells.

In addition, VEGF expression was also found in this study to be positively correlated with clinical stage of OSCC and LN metastasis. Similar results were reported in previous studies in HN cancer and OSCC [35–37]. This correlation may be attributed to the role of VEGF as a selective mitogen for vascular ECs that induce tumor angiogenesis by promoting the differentiation and proliferation of ECs. Then, VEGF guides the angiogenic sprouts to expand the primary plexus and vascularize the growing tumor that facilitate tumor expansion and invasion [38]. Additionally, VEGF was also reported to induce vasodilatation and increases vascular permeability. This makes the newly-formed capillaries more penetrable by tumor cells than normal vessels, consequently, increasing the risk of metastasis [39].

The direct correlation between HIF-1α and local recurrence is supported by the results of Katsuta et al. [40], Schrijvers et al. [41], in esophageal and glottic laryngeal carcinoma respectively. These results may reveal the role of HIF-1α in attaining an aggressive phenotype of the tumor cells that have the ability for invasion, metastasis and local recurrence.

Histologically, the localized HIF-1α expression around keratinized and necrotic areas of the tumor or in regions distal to the nearest blood vessel appeared to corroborate with the observations of previous studies [9,42]. This supports the hypothesis that HIF-1α expression is induced mainly by hypoxia and the presence of blood vessels in the tumor stroma does not necessarily ensure that a region of tumor is receiving appropriate perfusion and oxygenation. This could be due to the tumor blood flow that may be transiently compromised in regions where microvessels are immature, deformed or occluded. Moreover, areas where cells beyond the diffusion distance of oxygen from microvessel may be subjected to chronic hypoxia that strongly induce cells to produce HIF-1α as an adaptive mechanism to this hypoxia [42,43].

These previous findings are also supported by the observations of a more recent study by Zhu et al. [44], who observed that HIF-1α protein and mRNA levels were hardly detected under normoxic condition, but were remarkably increased at hypoxic condition analyzed by using Western blot and quantitative transcriptase-polymerase chain reaction (RT-PCR).

Furthermore, the diffuse HIF-1α expression observed in some cases of OSCC irrespective with the distance from the closest blood vessel, may be explained by the hypothesis that HIF-1α production is not only stimulated by hypoxia, but also with other signals as growth factors as well as the activation of other oncogenic signal transduction pathways that may play a role in upregulation of HIF-1α expression in addition to hypoxia [45].

Additionally, the nuclear expression of HIF-1α was more predominant and intense in advanced stages and increased degree of malignancy of OSCC in this study. These observations were in accordance with previous studies made by Ogane et al. [46] and Zhu et al. [44], who evaluated HIF-1α expression in SCC focusing on the nuclear pattern in comparison to the cytoplasmic one. They found that nuclear pattern predominance is considered to be strongly related to the activated status of HIF-1α as it becomes trans-located to the nucleus to dimerize with HIF-1β subunit to form HIF-1 molecule that may control many pathways involved in aggressive behavior of the tumors [47].

The significant correlation of HIF-1α expression with the histopathological grade of OSCC in the
current study is supported by the results of previous studies that revealed similar findings in OC [7,48]. This may indicate that HIF-1α upregulation occurs well in early carcinogenesis and an essential step before acquiring invasive phenotype. Emerging experimental evidence indicates that hypoxic HIF-1α expression plays important role in the regulation of phenotype and function of cancer stem cells (CSCs) by inducing the activation of signature genes of CSCs that enhance self-renewal capacity and maintenance of undifferentiated state of these cells [49].

In this study, only two cases of OSCC revealed no positivity of HIF-1α immunoreactivity. At present, there is no certain explanation for this finding. However, the prolonged fixation of tissue specimens is known to substantially compromise antigen detection, so that failure to stain these cases might be artificial. Otherwise, this negativity could be also explained that HIF-1α protein expression in these cases was at levels below the limits of detection by IHC methodology [50].

In the present study, VEGF expression was found to be significantly upregulated with increased malignant criteria of OSCC, as it was markedly higher in poorly-differentiated OSCC compared to well-differentiated cases. However, VEGF reactivity was not positive or very weak in the normal epithelium. Similar findings were reported by Macluskey et al. [11], and Shang et al. [51], who observed a significant increase in VEGF expression during the transition from normal oral mucosa through different degrees of dysplasia. These findings suggest the hypothesis that VEGF is strongly involved in angiogenesis and it is essential in the development of OSCC.

In the current study, HIF-1α and VEGF immunoreactivities were also observed in the vascular ECs of the tumor stroma and HIF-1α showed significant correlation with tumor angiogenesis measured by VEGF staining. These findings were in a line with a previous study that found that the endothelial HIF-1α mediates pathway for VEGF expression and deletion of HIF-1α in ECs disrupts an autocrine loop necessary for hypoxic induction of VEGFR-1 and VEGFR-2 signaling by preventing the binding of HIF-1 to an enhancer element located in VEGFR-1 and the post-transcriptional regulation of VEGFR-2 by HIF-1. Thus, this will result in decreased endothelial proliferation and tube formation in vitro leading to severely impaired tumor angiogenesis and reduced tumor growth in vivo [52].

Accordingly, all the previous observations of this study shed a light on the importance of TME changes, specifically hypoxia and angiogenesis, in controlling the behavior of OSCC. This confirms the hypothesis that the adaptive pathways to hypoxia as HIF-1α and VEGF expressions and their upregulation may play a crucial role in tumor angiogenesis, progression and consequently, controlling the behavior of OSCC.

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