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ORIGINAL ARTICLE

Retrospective Study

Hypoxia-inducible factor-1α at the invasive tumor front in oral squamous cell carcinoma

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Abstract

BACKGROUND

Hypoxia in oral cancer promotes tumoral invasion by inducing epithelial-mesenchymal transition, leading to aggressive tumor progression.

AIM

To characterize the expression of hypoxia-inducible factor 1-alpha (HIF-1α) at the invasive tumor front (ITF) in comparison to tumor islands (TI) in oral squamous cell carcinoma (OSCC) and to explore its relationship with E-cadherin and Vimentin expression.

METHODS

Thirty-eight cases of OSCC and five cases of normal oral mucosa (NOM) were

included in this study. The ITF was identified based on the region and immune expression of AE1/AE3. Immunohistochemistry was performed to assess the expression of HIF-1α, Vimentin, and E-cadherin. The immunostaining was analyzed using an immunoreactive score, and the results were illustrated using immunofluorescence.

RESULTS

HIF-1α expression was significantly higher in the TI region compared to the ITF region (P = 0.0134). Additionally, a significant difference was observed between TI and NOM (P = 0.0115). In the ITF regions, HIF-1α expression showed a significant correlation with Vimentin expression, with higher levels of HIF-1α associated with increased Vimentin expression (P = 0.017).

CONCLUSION

Based on the results of this study, HIF-1α appears to play a distinct role in OSCC tumor progression, underscoring the importance of exploring hypoxia-driven changes in cellular phenotype at the ITF of OSCC. Further research is needed to better understand their impact on OSCC prognosis.

Key Words: Hypoxia-inducible factor-1α; Oral squamous cell carcinoma; Immunohistochemistry; Vimentin; E-cadherin

Core Tip: This study aimed to characterize the expression of hypoxia-inducible factor 1-alpha (HIF-1α) at the invasive tumor front (ITF) in comparison to tumor islands in oral squamous cell carcinoma (OSCC) and to explore its relationship with E-cadherin and Vimentin expression. Based on the results of this study, HIF-1α appears to play a distinct role in OSCC tumor progression, underscoring the importance of exploring hypoxia-driven changes in cellular phenotype at the ITF of OSCC.

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INTRODUCTION

According to GLOBOCAN 2022 data from the International Agency for Research on Cancer, head and neck cancers accounted for approximately 930000 new cases and 500000 deaths globally, representing around 5% of all cancer cases[1]. Oral squamous cell carcinoma (OSCC) constitutes approximately 90% of all oral cancers. The complexity of OSCC is underscored by its multifactorial etiology, which significantly alters the tumor microenvironment (TME), making it a critical focus of OSCC research. Although the molecular interactions within the OSCC TME have been extensively studied, they remain only partially elucidated[2,3].

Hypoxia is a common feature of solid tumors, allowing them to adapt and survive in the TME under conditions of reduced oxygen concentration, which would typically lead to cell death. Hypoxia in tumors is primarily mediated by hypoxia-inducible factors (HIF), namely HIF-1, HIF-2, and HIF-3, which play critical roles in tumorigenesis, progression, and response to cancer therapy[4,5]. Among these, HIF-1 is a heterodimeric protein composed of two subunits, HIF-1α and HIF-1β. HIF-1α is particularly significant, as it primarily drives the transcription of numerous genes associated with cancer[6]. HIF-1α expression has been observed in various malignancies, including renal[7], bladder[8], colorectal[9], breast[10], and head and neck cancers[11]. Notably, in some of these carcinomas, HIF-1α expression has been significantly linked to survival outcomes and prognosis[8,10,12].

The invasive tumor front (ITF) is the site where the initial tumor cells detach from the epithelium and penetrate the connective tissue. The prognostic significance of the ITF in OSCC has been recognized, with studies highlighting its distinct characteristics compared to central tumor regions[13-15]. At this stage of tumor progression, individual and/or collective clusters of cells begin to migrate and invade the surrounding tissue[16]. In this context, neoplastic cells in the ITF region can sometimes be characterized by features of epithelial-mesenchymal transition (EMT). During EMT, these cells progressively downregulate or redistribute their epithelial-specific proteins, such as E-cadherin and cytokeratins, associated with apical and basolateral tight and adherens junctions. Concurrently, they begin to re-express mesenchymal markers, including vimentin and N-cadherin[17-19]. In malignant neoplasms, tumorigenesis-associated EMT encompasses additional crucial characteristics that can significantly enhance the cells' malignancy, promoting local tumor invasion and metastasis[20]. The association between HIF-1α and EMT, along with the mechanisms by which hypoxia influences this process, has been investigated but remains inadequately understood[20-22]. Specifically for OSCC, Ishida *et al*[23] demonstrated that hypoxia induces EMT in OSCC cell lines through the activation of Notch signaling. Under hypoxic conditions, a reduction in E-cadherin expression, increased motility and invasiveness, and upregulation of Notch pathway components were observed, underscoring the critical role of Notch signaling in hypoxia-driven EMT.

Despite extensive research into the role of hypoxia in various cancers, including OSCC, the specific interplay between HIF-1α expression at the ITF and changes in epithelial and mesenchymal markers presents an intriguing area for further study. Therefore, this study aims to investigate the expression of HIF-1α at the ITF of OSCC and its potential associations with the expression of E-Cadherin and Vimentin.

MATERIALS AND METHODS

Tissue samples

This study was approved by the Ethics Committee of the School of Dentistry of the Universidad de la República (Uruguay) Exp. 091900-000178-20. This retrospective study randomly selected 38 formalin-fixed, paraffin-embedded cases of OSCC and five cases of normal oral mucosa from the repository of the School of Dentistry, Universidad de la República (Uruguay).

Immunohistochemistry technique

For the immunohistochemical reactions, 3 μm sections of each case were subjected to antigen retrieval using a heat-induced epitope retrieval solution (Reveal Decloaker, RTU; Biocare Medical). To block endogenous peroxidase activity, the sections were treated with 0.9% hydrogen peroxide for 5 minutes. The tissue samples were then incubated for 60 minutes with primary antibodies against AE1 and AE3 (Clon: BSB5433, monoclonal antibody, Bio SB, Santa Barbara, CA, United States, 1: 500 dilution), HIF-1α (Clone: EP 18, monoclonal antibody, Bio SB, Santa Barbara, CA, United States, 1:200 dilution), E-cadherin (Clone: EP6, monoclonal antibody, Bio SB, Santa Barbara, CA, United States, 1: 500 dilution), or Vimentin (Clone: V9, monoclonal antibody, Bio SB, Santa Barbara, CA, United States, 1:100 dilution). Following this, the sections were incubated for 40 minutes each with a biotinylated anti-mouse/anti-rabbit antibody and a streptavidin-horseradish peroxidase (HRP) complex (Immuno Detector Biotin Link and HRP Label; Bio SB). Negative controls were prepared by omitting the primary antibody, while breast carcinoma, normal epithelial tissue, and normal mesenchymal tissue were used as positive controls. The immunoreactive products were visualized using a 3,3'-diaminobenzidine-H_2O_2 substrate (Biocare Medical), and the sections were counterstained with Harris hematoxylin.

Immunohistochemical analysis

All slides were digitized using a digital scanner (Motic Asia, MoticEasyScan One, Hong Kong) and analyzed digitally with Pathomation software (Pathomation BV, Belgium). For each case, when present, three hotspots were selected for evaluating HIF-1α expression, focusing specifically on areas at the ITF and within TI. The ITF was defined as the leading edge of the tumor, characterized by neoplastic cells infiltrating the surrounding connective tissue and dissociating from the primary tumor mass, whereas TI was defined as clusters of neoplastic cells located deeper within the tumor mass. These regions were highlighted by the positive immunoexpression of AE1/AE3. Subsequently, the same ITF zones were analyzed for E-cadherin and Vimentin immunoreactivity. Samples were assessed using an immunoreactive score (IRS), combining the percentage of positive cells (0–5) with staining intensity (0–3)[24,25]. The IRS, ranging from 0 to 15, was calculated by multiplying these two scores. The percentage of positive cells was scored from 0 (0%) to 5 (≥ 80%), while staining intensity was scored from 0 (no staining) to 3 (strong staining intensity). In this study, immunostaining was categorized as follows: Negative (-, IRS 0), weak-to-moderate (+, IRS 1–7), and strong (++, IRS ≥ 8). The weak-to-moderate category includes samples with low to moderate staining intensity (IRS 1–7). The strong category (IRS ≥ 8) indicates a robust immunoreactivity. In this study, two observers evaluated the immunohistochemical staining results together by reviewing digital images of the samples. Any discrepancies in the assessment were resolved through discussion and consensus.

Immunofluorescence technique

To illustrate the expression and co-expression of the studied markers, select cases were chosen for tissue analysis using immunofluorescence. Briefly, 3 μm sections of each sample were deparaffinized, hydrated, and treated with Reveal Decloaker thermal retrieval solution (RTU; Biocare Medical, Pacheco, CA, United States) in a water bath to expose antigenic epitopes. Endogenous peroxidases were blocked with 0.9% hydrogen peroxide for 5 minutes, and global blocking was performed with 2% Bovine Serum Albumin (BSA, Capricorn, Germany) for 20 minutes at room temperature (RT). For double labeling, the samples were first incubated with the primary antibody HIF-1α (Clone: EP18, Bio SB, Santa Barbara, CA, United States) at a 1:100 dilution and DAPI (4',6-diamidino-2-phenylindole, Biotium, Fremont, CA, United States) at a 1:1000 dilution for 60 minutes at RT in the dark. Then, the samples were incubated with a biotinylated secondary antibody, followed by a streptavidin-peroxidase complex, both for 40 minutes at RT in the dark (Mouse/Rabbit Immuno Detector Biotin Link, Bio SB, Santa Barbara, CA, United States). Next, they were incubated for 10 minutes with CF® 488 tyramide (Biotium, Fremont, CA, United States) at RT in the dark. After appropriate washes with PBS 1 ×, a second incubation was performed with the primary antibody E-cadherin at a 1:500 dilution (Clone: EP6, Bio SB, Santa Barbara, CA, United States) or Vimentin at a 1:50 dilution (Clone: V9, Bio SB, Santa Barbara, CA, United States) for 60 minutes at RT in the dark. Following incubation with these primary antibodies, the sections were incubated again with a biotinylated secondary antibody followed by a streptavidin-peroxidase complex under the same conditions as before. They were then incubated with CF® 647 tyramide (Biotium, Fremont, CA, United States) at a 1:300 dilution for 10 minutes at RT in the dark. Finally, all samples were dehydrated and mounted with Glas™ Mounting Medium (Tissue-Tek, Sakura Finetek, United States). The samples were observed and imaged using a Zeiss LSM 800 confocal microscope, and the

images were analyzed using FIJI software.

Statistical analysis

A descriptive analysis was performed based on mean and standard deviation. Comparative analyses between groups were evaluated using the Mann-Whitney test for the variables of HIF-1α expression normal oral mucosa, TI, or at the ITF of OSCC cases. Additionally, correlation studies were conducted between the protein expression variables, and Pearson's χ^2 tests were used since the continuous variables were categorized. For all analyses, statistical significance was determined using a P value ≤ 0.05 and the P-values of significance are indicated in each figure. IBM SPSS software was used for all statistical analyses, and GraphPad software was used for generating the graphs.

RESULTS

HIF-1α expression was localized in both the nucleus and cytoplasm of the neoplastic cells of positive OSCC cases. In normal epithelial cells, the cytoplasmic expression appeared to be weaker. HIF-1α expression in the selected TI regions was negative in 4 (10.5%) cases, and in the selected ITF regions, it was negative in 9 (23.7%) cases of OSCC. Among the positive cases, in the TI regions, 12 (31.6%) cases showed weak-to-moderate expression, and 22 (57.9%) cases exhibited high expression. In contrast, in the ITF regions, 17 (44.7%) cases demonstrated weak-to-moderate expression, while 12 (31.6%) cases showed high expression. For cases of normal oral mucosa, all cases presented weak-to-moderate HIF-1α expression (Table 1).

Comparison of HIF-1α expression in normal oral mucosa, tumor islands of OSCC, and the ITF of OSCC

HIF-1α expression was significantly higher in TI compared to the ITF regions (P = 0.0134). Additionally, a significant difference in HIF-1α expression was observed between the TI and normal mucosa (P = 0.0115). However, no significant difference in HIF-1α expression was found between the ITF regions and normal oral mucosa (Figure 1A-C). HIF-1α expression in TI both cytoplasmic and nuclear is further illustrated in the images obtained *via* confocal microscopy (Figure 1D-G).

Correlation of HIF-1α expression with E-cadherin and vimentin expressions in the ITF

In the ITF regions, HIF-1α expression showed a significant correlation with Vimentin expression, with higher levels of HIF-1α associated with increased Vimentin expression (P = 0.017) (Figure 2). However, no significant correlation was found between HIF-1α expression and E-cadherin expression (P = 0.287) (Figure 3). The images obtained *via* confocal microscopy illustrate the co-localization of HIF-1α and vimentin expression (Figure 2D-F), as well as the expression of HIF-1α with E-cadherin (Figure 3D-F).

DISCUSSION

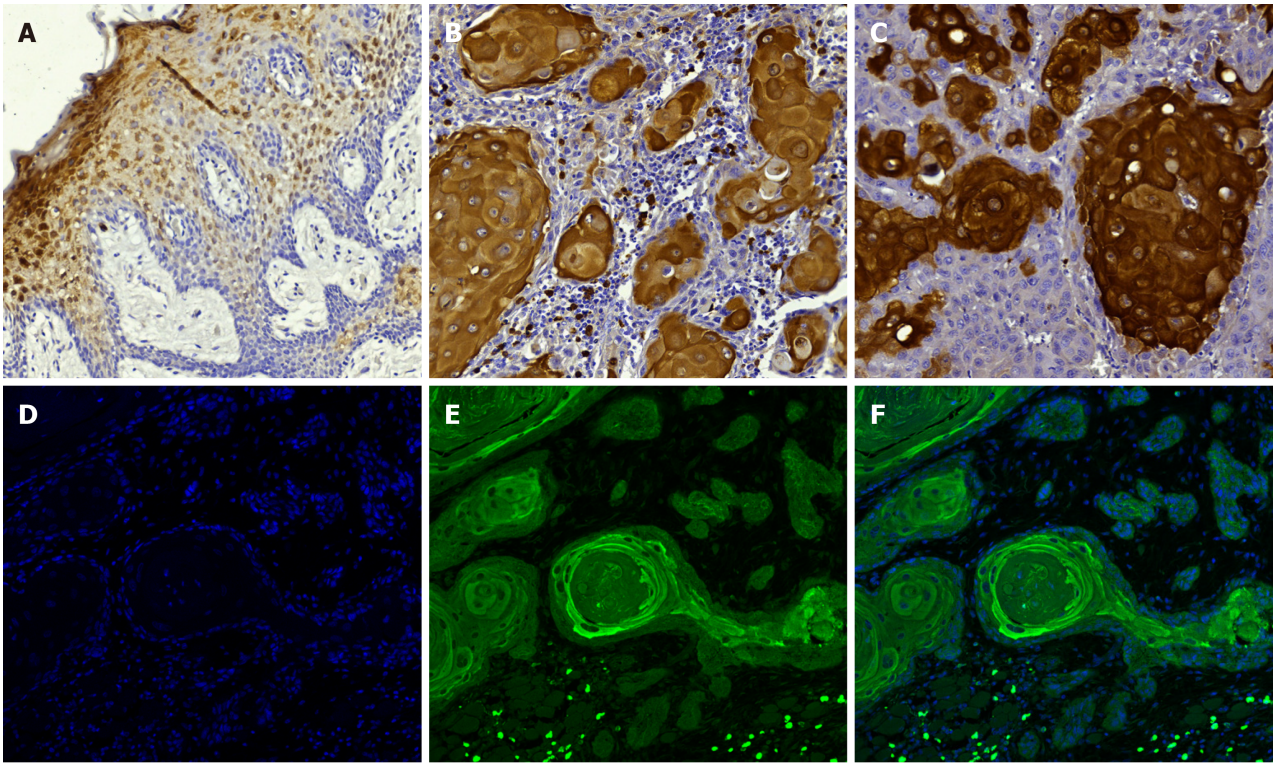
Understanding the molecular mechanisms underlying OSCC is crucial for advancing our knowledge of the disease and identifying novel molecular targets. Hypoxia, a common feature in solid tumors, plays a significant role in tumor biology by enabling cancer cells to adapt and thrive in low-oxygen environments[2]. The ITF of OSCC is a region critical for tumor spread characterized by notable changes in marker expression[26]. Investigating how HIF-1α expression at ITF correlates with these markers can provide novel insights into the molecular mechanisms driving OSCC. This study showed that HIF-1α expression differed significantly between the TI and the ITF regions, and between TI and normal mucosa. At the ITF regions, higher HIF-1α expression was notably associated with increased levels of Vimentin, suggesting a potential link between hypoxic response and mesenchymal marker expression in OSCC.

Tumor aggressiveness is often linked to mutations in oncogenes or tumor suppressor genes. Hypoxic conditions in pathological situations may exacerbate genomic instability by increasing mutation rates, contributing to both local and systemic cancer progression, resistance to therapy, and poor patient outcomes[27]. Initially, HIF-1 was recognized for its role in responding to low oxygen levels. However, it has become evident that HIF-1 activity can also be influenced by other factors such as oncogene activation (*e.g.*, Rat Sarcoma, Sarcoma, and Phosphoinositide 3-Kinase) or the loss of tumor suppressors (*e.g.*, von Hippel–Lindau and Phosphatase and Tensin Homolog)[28]. In oxygen-deprived environments, the hydroxylation of HIF-1α is inhibited, leading to its stabilization. Once stabilized, HIF-1α translocates to the nucleus, where it dimerizes with HIF-1β to form HIF-1, which activates a range of target genes involved in both physiological and pathological processes[28]. HIF-1 functions as a transcriptional activator, binding to the promoters or enhancers of over 60 hypoxia-inducible genes, including those encoding erythropoietin, vascular endothelial growth factor (VEGF), and various enzymes involved in glucose, iron, and nucleotide metabolism[29,30].

Regarding hypoxia in head and neck cancer, some studies have reported on the expression of HIF-1α in oral cancer and its association with different outcomes. Kang *et al*[31] showed that HIF-1α and VEGF mRNA were significantly expressed in tongue squamous cell carcinoma (TSCC) but barely detected in adjacent normal tissues. Additionally, HIF-1α overexpression was significantly associated with tumor size, lymphatic metastasis, and histological differentiation, as well as poorer overall and disease-free survival rates, suggesting that HIF-1α overexpression may indicate a poor prognosis and could serve as a prognostic marker for TSCC. Eckert *et al*[32] demonstrated that strong HIF-1α expression was linked to reduced disease-specific survival in 82 OSCC patients, with a 3.5-fold higher risk of tumor-related death compared to

Table 1 Immunoexpression of hypoxia-inducible factor 1-alpha in the oral squamous cell carcinoma cases studied according to the evaluated region, *n* (%)

	Negative	Weak-to-moderate	High	Total
Invasive tumor front	9 (23.7)	17 (44.7)	12 (31.6)	38
Tumor islands	4 (10.5)	12 (31.6)	22 (57.9)	38
Total	13	29	34	76



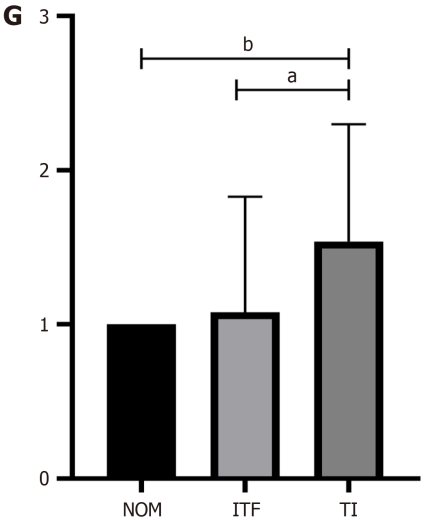


Figure 1 Expression of hypoxia-inducible factor 1-alpha. A: Immunohistochemical expression of hypoxia-inducible factor 1-alpha (HIF-1α) in the normal oral mucosa (NOM); B: Invasive tumor front (ITF); C: Tumor islands (TI). HIF-1α expression was significantly different between the TI and ITF regions (P = 0.0134). Additionally, a significant difference was observed between TI and NOM (P = 0.0115). (Immunohistochemistry, 20 × magnification); D: DAPI-stained nuclei (blue); E: HIF-1α expression (green); F: Merged image showing HIF-1α localization in both the cytoplasm and nucleus of neoplastic cells (Immunofluorescence, 20 × magnification); G: HIF-1α expression was significantly different between the TI and ITF regions (^aP = 0.0134). Additionally, a significant difference was observed between TI and NOM (^bP = 0.0115).

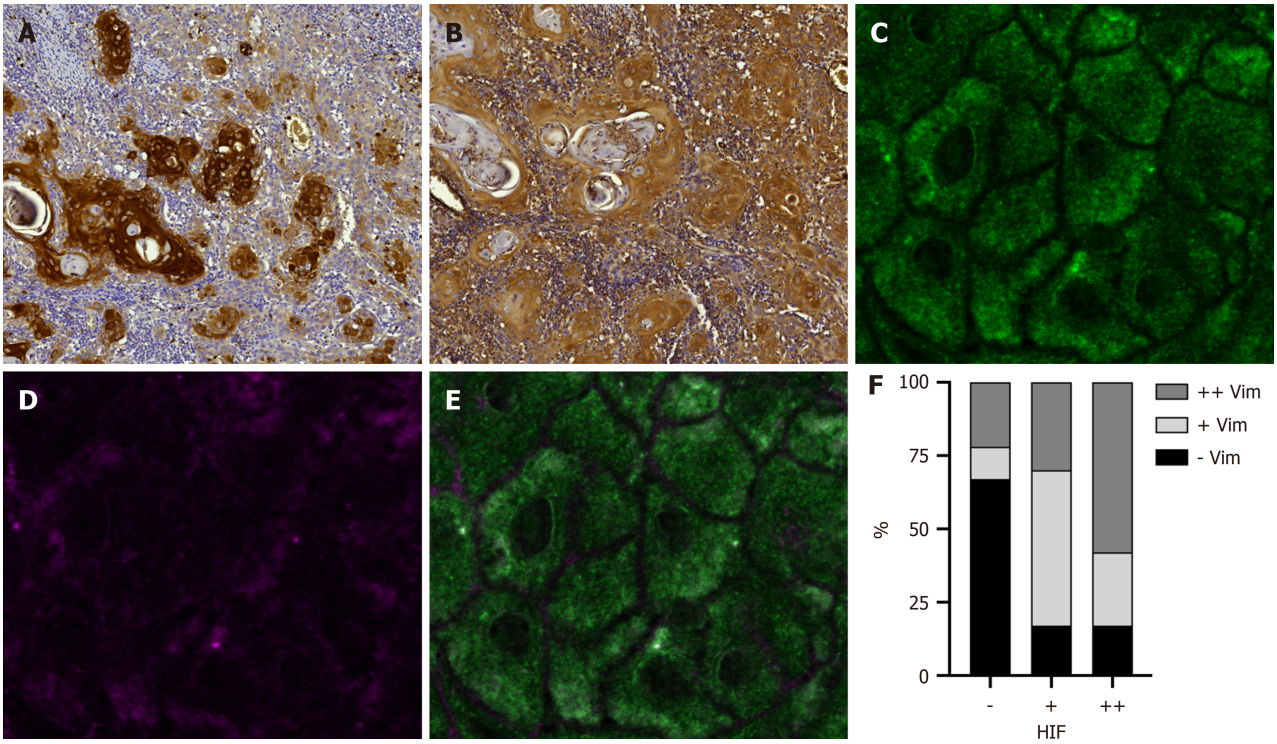


Figure 2 Correlation of hypoxia-inducible factor 1-alpha expression with vimentin expression. A: Immunohistochemical expression of hypoxia-inducible factor 1-alpha (HIF-1α); B: Vimentin expression, which was significantly correlated with HIF-1α (P = 0.017). (Immunohistochemistry, 20 × magnification); C: HIF-1α expression (green); D: Vimentin expression (magenta); E: Colocalization of HIF-1α and vimentin (white) in images obtained *via* confocal microscopy (Immunofluorescence, 63 × magnification); F: Vimentin expression was significantly correlated with HIF-1α (P = 0.017).

those with negative or weak HIF-1α expression. These results highlight the importance of further elucidating HIF-1α expression in OSCC.

In this study, significant differences in HIF-1α expression were found between TI and the ITF regions, as well as between TI and normal mucosa. These findings suggest that HIF-1α may play a role in the molecular changes occurring at different stages of tumor progression, with potentially higher expression in TI reflecting the distinct metabolic and hypoxic demands of these areas compared to the ITF regions. The histopathological features of OSCC can vary significantly within the same tumor, with cells exhibiting different characteristics depending on their location within the tumor[33]. In contrast, the absence of a significant difference in HIF-1α expression between the ITF regions and normal mucosa may indicate that other factors could be influencing HIF-1α expression in these regions. This is particularly relevant if the neoplastic cells are in the early stages of carcinogenesis, where HIF-1α expression might not yet be significantly elevated compared to normal cells. Although it has been reported that HIF-1α protein is absent in normal human tissues[34,35], it has also been demonstrated that HIF-1α can be present under normoxic conditions in certain normal tissues, likely due to physiological oxygen tension maintaining a low level of HIF-1α to support tissue homeostasis and the basal induction of genes necessary for meeting cellular energy needs[36]. It is possible to infer that more pronounced differences in HIF-1α expression will emerge in later stages of OSCC development, as evidenced by the significantly higher expression of HIF-1α observed in TI in this study. This suggests that HIF-1α expression may become more distinct as the tumor progresses, reflecting its increasing role in tumor-specific processes and further differentiating it from normal epithelial tissues. Furthermore, the differential expression of HIF-1α in TI and ITF regions may have implications for biomarker development, highlighting its potential as a prognostic indicator or therapeutic target. HIF-1 inhibitors modulate HIF-1 expression or function through various molecular mechanisms, including the suppression of HIF-1α mRNA transcription, inhibition of protein synthesis, prevention of HIF-1α stabilization, disruption of HIF-1α/HIF-1β dimerization, interference with HIF-1 binding to DNA, and suppression of its transcriptional activity[37]. Several HIF-1 inhibitors have been developed and assessed as potential anticancer agents in both preclinical and clinical settings, primarily for the management of advanced or treatment-resistant cancers, however, further insights are needed to identify tumors reliant on this pathway, suitable biomarkers, and indicators of effective HIF inhibition[38-40].

Interestingly, this study found that in neoplastic cells, HIF-1α expression was predominantly observed in both the nucleus and the cytoplasm in at least one region of the OSCC tissue samples. In normal epithelial cells, the cytoplasmic expression appeared weaker. Some studies have demonstrated a relationship between the nuclear expression of HIF-1α and significant outcomes in OSCC. Zhu *et al*[41] found that HIF-1α was strongly linked to T stage, lymph node involvement, histologic differentiation, and micro vessel density. Patients with positive HIF-1α nuclear staining had significantly poorer overall and disease-free survival rates. Lin *et al*[42] found that nuclear HIF-1α expression increased from normal oral mucosa to oral epithelial dysplasia, suggesting that elevated HIF-1α is an early marker of oral carcinogenesis. In OSCC samples, higher nuclear HIF-1α expression was significantly associated with advanced tumor and nodal stages, as well as overall clinical staging. It could be inferred that strong cytoplasmic staining indicates a significant

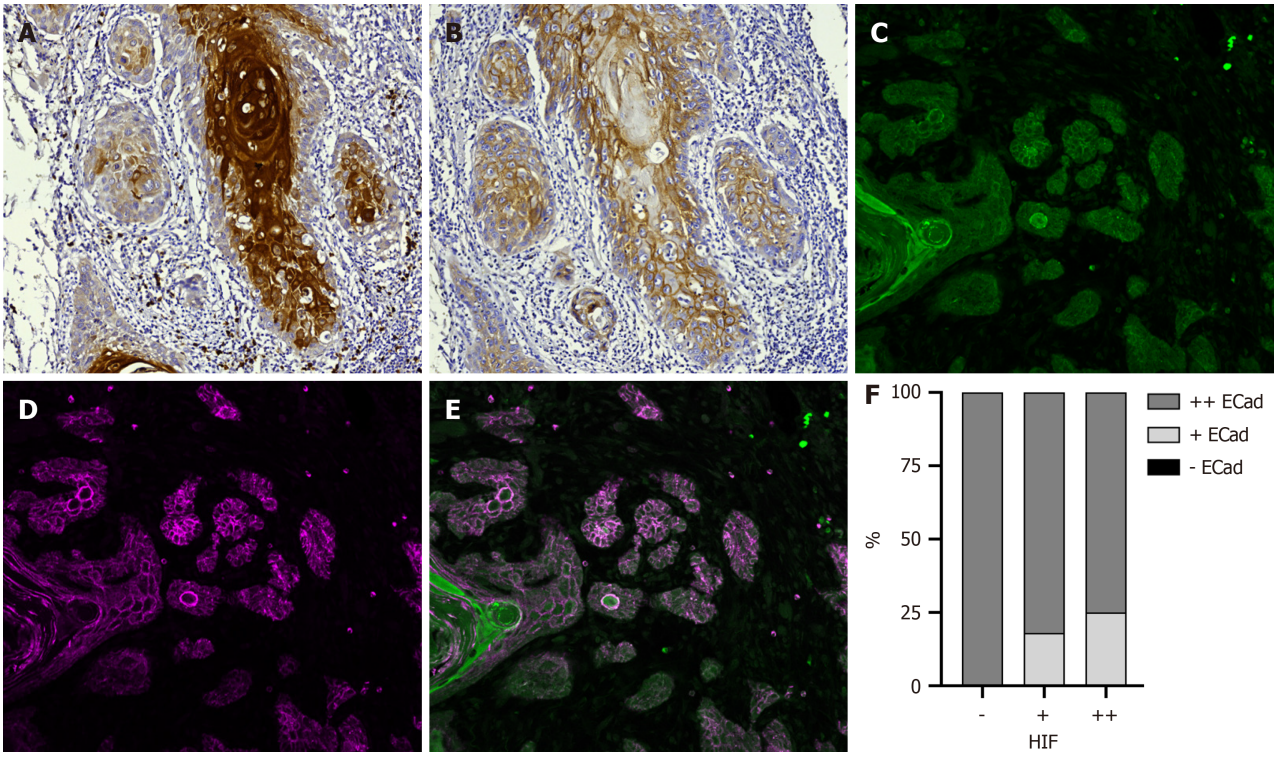


Figure 3 Correlation of hypoxia-inducible factor 1-alpha expression with E-cadherin expression. A: Immunohistochemical expression of hypoxia-inducible factor 1-alpha (HIF-1α); B: E-cadherin expression. No significant correlation was found between HIF-1α and E-cadherin expression (P = 0.287). (Immunohistochemistry, 9 × magnification); C: HIF-1α expression (green); D: E-cadherin expression (magenta); E: Colocalization of HIF-1α and E-cadherin (white) in images obtained *via* confocal microscopy (Immunofluorescence, 20 × magnification); F: No significant correlation was found between HIF-1α and E-cadherin expression (P = 0.287).

accumulation of HIF-1α, which might suggest active or prolonged hypoxia in the tissue.

To address the main objective of this study, the expression of HIF-1α in the ITF regions was evaluated in conjunction with the immune expression of E-Cadherin and Vimentin. A significant correlation was observed between HIF-1α and Vimentin expression, with higher levels of HIF-1α associated with increased Vimentin expression in ITF regions. However, no significant correlation was found between HIF-1α expression and E-Cadherin expression. Previous *in vitro* studies have already demonstrated a reduction in E-Cadherin expression under hypoxic conditions in OSCC cells[43] and ovarian carcinoma cells[44]. Domingos *et al*[43] observed that hypoxic conditions led to a significant decrease in E-cadherin mRNA levels, which was accompanied by an increase in the migratory capacity of OSCC cells. These findings differ from those observed in the present study. However, it is important to note that these previous studies primarily evaluated E-Cadherin mRNA expression, which may differ with the protein expression assessed in this study. Additionally, different molecular mechanisms may regulate E-Cadherin expression in the context of malignant neoplasms, independent of HIF-1α expression[45].

Research has indicated a link between hypoxia and EMT; for example, HIF-1α has been shown to directly activate Twist1, which subsequently regulates the expression of EMT-related genes, including E-cadherin, Vimentin, and N-cadherin, thereby facilitating EMT[46,47]. Vimentin is a marker of mesenchymal cells, and its upregulation may suggest that epithelial cells are acquiring mesenchymal characteristics, which can lead to increased motility and invasiveness. The expression levels of this marker may indicate a phenomenon known as EMT, which signifies the shift towards a more aggressive and invasive phenotype[48]. Based on the results of this study, it can be observed that although changes in HIF-1α levels do not significantly affect E-Cadherin expression in the ITF, they are associated with higher Vimentin expression. In this context, it can be inferred that cells in regions with more severe hypoxic conditions may exhibit a more mesenchymal phenotype, possibly indicating the initiation of an EMT process. In this context, previous studies have reported a positive correlation between HIF-1α expression and Vimentin expression in various cancer types, such as colorectal cancer[49] and hepatocellular carcinoma[50].

This study has limitations that should be considered. First, the relatively small number of OSCC cases included may limit the generalizability of the findings. Additionally, the study relied solely on immunohistochemistry to assess protein expression, which, while informative, does not provide a comprehensive analysis of molecular mechanisms or dynamic changes in protein levels. Furthermore, the study focused on a limited set of markers, and incorporating additional markers related to EMT could offer a more complete understanding of the processes involved in OSCC progression. Future research with larger sample sizes, a broader range of biomarkers, and complementary techniques will be essential to validate and expand upon these findings.

CONCLUSION

In conclusion, this study presents important findings regarding HIF-1α expression in OSCC. We observed a significant difference in HIF-1α expression between TI and both the ITF regions and normal mucosa, probably indicating a distinct role for HIF-1α in tumor progression. Notably, HIF-1α expression correlated significantly with Vimentin levels in the ITF regions, suggesting a link between hypoxia and a more mesenchymal phenotype. The observed correlation between HIF-1α and Vimentin underscores the importance of exploring hypoxia-driven changes in cellular phenotype. The results of this study highlight the need for larger cohort studies to validate the clinical relevance of HIF-1α in OSCC, as well as investigations into its integration with other molecular markers to potentially improve prognostic accuracy. Further research with expanded sample sizes and additional biomarkers is essential to deepen our understanding of these relationships and their implications for OSCC progression and prognosis.

FOOTNOTES

Author contributions: Silveira FM contributed to conceptualization, methodology, investigation, data analysis, writing–original draft; Schuch LF contributed to methodology, investigation, data analysis, writing–original draft; Pereira-Prado V contributed to investigation, writing–original draft; Sicco E contributed to methodology, investigation, data analysis, writing–original draft; Molina-Frechero N, López-Verdín S, Palacio-Gastelum MG contributed to writing–review and editing; Arocena M contributed to conceptualization, methodology, writing–review and editing; Niklander S contributed to resources, writing–review and editing; Bologna-Molina R contributed to conceptualization, methodology, resources, writing–review and editing, supervision.

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Informed consent statement: Informed consent statement was obtained for this study.

Conflict-of-interest statement: The authors declare do not have any Conflict of Interest.

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