RESEARCH ARTICLE

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Enhancement of mRNA Vascular Endothelial Growth Factor-A (VEGF-A) Induced Anemia in Oral Squamous Cell Carcinoma (OSCC)

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is a malignant epithelial neoplasm that can lead to mechanical disturbances and malnutrition, potentially causing anemia. Vascular endothelial growth factor-A (VEGF-A) plays a crucial role in angiogenesis and vascularization, which may impact anemia in OSCC patients. This study aimed to investigate the differential mRNA expression of VEGF-A in OSCC patients with and without anemia.

Method: This cross-sectional observational study analyzed 39 formalin-fixed paraffin-embedded (FFPE) samples from OSCC patients (13 with anemia, 26 without anemia). VEGF-A mRNA expression was quantified using qPCR with GAPDH as a reference gene. The Livak method was used to determine VEGF-A expression levels. Data were analyzed using GenEX MultiD 6.0 software, and statistical significance was assessed using independent T-tests.

Results: VEGF-A mRNA expression in OSCC patients varied based on anemia status (1.94-fold), tumor size (1.44-fold), histological grade (1.21-fold), and anemia severity (1.30-fold). Significant differences in VEGF-A mRNA expression were observed for anemia status and tumor size ($p < 0.05$), while histological grading and anemia severity showed no significant differences.

Conclusion: This study demonstrates a significant difference in VEGF-A mRNA expression in OSCC patients with anemia, particularly in relation to tumor size. These findings contribute to our understanding of the relationship between VEGF-A expression and anemia in OSCC, potentially informing future diagnostic and therapeutic strategies.

INTRODUCTION

Oral cancer represents a significant global health challenge, with an estimated 377,713 new cases and 177,757 deaths worldwide in 2020, according to GLOBOCAN data. In Indonesia, the prevalence of oral cancer has shown a concerning upward trend, rising from 1.4 per 1000 population in 2013 to 1.79 per 1000 population in 2018 [1,2]. Oral squamous cell carcinoma (OSCC) accounts for approximately 90% of oral cancer cases, primarily affecting the tongue, palate, lips, and floor of the mouth.

OSCC is a complex, multifactorial malignancy with various risk factors, including tobacco use, alcohol consumption, betel quid chewing, human papillomavirus (HPV) infection, and poor oral hygiene [3–5]. The development of OSCC involves genetic mutations that disrupt normal cellular functions, leading to alterations in the immune system and inflammatory responses [6,7]. These changes can result in shifts in blood cell populations, with increased neutrophils and monocytes but decreased lymphocytes, depending on the severity of the patient's condition [8,9].

Anemia is a common complication in cancer patients, significantly impacting their prognosis and quality of life [10,11]. In OSCC patients, anemia can lead to hypoxic conditions within the tumor microenvironment, triggering the expression of hypoxia-inducible factor-1α (HIF-1α). This, in turn, stimulates the production of vascular endothelial growth factor (VEGF), a key player in angiogenesis and tumor progression. VEGF-A, a member of the VEGF family, is particularly crucial in vasculogenesis and angiogenesis, influencing both normal and pathological vascular development [10].

Previous studies have demonstrated that VEGF overexpression correlates with poor survival in various cancer types, including ovarian, lung, and colorectal cancers [10]. However, the specific relationship between VEGF-A mRNA expression, anemia, and OSCC progression remains understudied. This gap in knowledge presents an opportunity to explore the potential role of VEGF-A as a prognostic marker and therapeutic target in OSCC [2,12].

The present study aims to investigate the overexpression of VEGF-A mRNA in OSCC patients with anemia using quantitative PCR (qPCR) techniques. By examining this relationship, we seek to elucidate the molecular mechanisms underlying the association between anemia and OSCC progression. Our findings may contribute to improved prognostic assessments and the development of targeted therapies for OSCC patients.

METHODS

Study design and sample collection

This observational-analytic study employed a cross-sectional design. Formalin-fixed paraffin-embedded (FFPE) samples were collected from oral squamous cell carcinoma (OSCC) patients at Prof. Dr. Margono Soekarjo Hospital between 2017 and 2020. Inclusion criteria were patients diagnosed with OSCC by histopathological examination, while exclusion criteria included patients who had received cancer therapy. Anemia was defined as hemoglobin levels < 12 g/dL for women and < 13 g/dL for men. Using purposive sampling, we selected 13 OSCC patients with anemia and 26 OSCC patients without anemia (control group). Clinical data, including complete blood count, tumor size, and histological assessment, were obtained from medical records.

RNA isolation and cDNA synthesis

RNA was isolated from FFPE samples using the Monarch® NEB kit (#T2010S) following the manufacturer's protocol. RNA purity (A260/A280 ratio) ranged from 1.8 to 2.0, with a minimum concentration of 2.2 ng/µL. Samples with higher concentrations were diluted to achieve uniform RNA input for cDNA synthesis. cDNA was synthesized using the ProtoScript® NEB kit (#E6300S/L). The reaction mixture (23 µL total) contained 2 µL d(T)23VN (50 µM), 10 µL M-MulV reaction mixture, 2 µL M-MulV enzyme mixture, 1 µL random primer mixture, 3 µL nuclease-free water, and 5 µL RNA sample. Synthesis was performed using a Veriti™ 96-Well Fast Thermal Cycler with the following program: 70°C for 5 minutes (denaturation), 25°C for 5 minutes, 42°C for 1 hour, and 80°C for 5 minutes (enzyme inactivation). cDNA samples were stored at -20C.

Quantitative real-time PCR

VEGF-A mRNA expression was quantified using an Applied Biosystem 7500 Real-Time PCR system, with GAPDH as a reference gene (13–17). Primer sequences were as follows: VEGF-A: Forward 5'-GCACCCATGGCAGAAGG-3', Reverse 5'-CTCGATGGATGGCAGTAGCT-3' (124 bp product). GAPDH: Forward 5'-AAGACGGGCGGAGAAACC-3', Reverse 5'-GTTGACTCCGACCTTCACCTT-3'. The 20 µL reaction mixture contained 10 µL 2×SensiFAST SYBR® Lo-ROX (#BIO-94005), 0.8 µL each of forward and reverse primers (10 µM), 6.4 µL nuclease-free water, and 2 µL cDNA (50 ng/µL). The PCR program consisted of 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Melt curve analysis was performed post-amplification.

Data analysis

Relative VEGF-A mRNA expression was calculated using the 2^-ΔΔCT method (Livak's method). Fold change values ≥ 1 indicated increased expression, while values < 1 indicated decreased expression. The relationship between anemia and clinical conditions in OSCC patients was analyzed using the Chi-square test. Differences in VEGF-A mRNA expression between anemic and non-anemic OSCC patients were assessed using an independent t-test. All statistical analyses were performed using GenEX MultiD 6.0 software, with $p < 0.05$ considered statistically significant. Graphs were generated using GraphPad Prism 9.

RESULTS

Sample's characteristics

A total of 39 samples of the Blood Assessment are presented in **Table 1**. There were 13 OSCC patients with anemia and 26 OSCC patients without anemia. The tumor size commonly found in the size of ≤ 5 cm histological grading samples is more on the low grade

than on the high grade. The anemia Severity of OSCC patients is shown in **Table 2**. OSCC patients with anemia are distinguished by the level of severity. OSCC patients with mild anemia in women were 6 people (46.2%), OSCC patients with anemia in men were 4 people (30.8%), and in OSCC patients with moderate anemia in 3 people (23%). The relevant data can be found in **Table 3**.

Figure 1 summarizes the comparative analysis of VEGF-A mRNA expression across distinct clinical subgroups of OSCC patients. Notably, VEGF-A expression was markedly elevated in patients with anemia relative to those without anemia, and was also significantly higher in cases with larger tumor volumes. In contrast, no statistically significant differences in VEGF-A expression were observed with respect to histological tumor grade or anemia severity classification. These findings underscore the association between VEGF-A upregulation and both anemia status and tumor size in OSCC, while suggesting limited correlation with tumor grade or anemia severity.

Table 1. Blood assessment (N=39)

Variables	Normal n (%)	Abnormal n (%)	(Mean ± SD)	p
Hemoglobin	13 (33.3%)	26 (66.6%)	12.5 ± 2.7	0.00*
Leukocytes	22 (56.4%)	17 (43.5)	19,060 ± 22,069	0.03*
Thrombocyte	35 (89.7%)	4 (10.2%)	330,153 ± 107,269	0.45
Eosinophil	16 (41%)	23 (58.3%)	8.7 ± 1.7	0.48
Lymphocytes	9 (23%)	30 (76.9%)	17.2 ± 10.2	0.38
Basophils	39 (100%)	0	0.35 ± 0.35	0.35
Monocytes	31 (79.4%)	8 (20.5%)	5.7 ± 2.05	0.21

Table 2. Clinical characteristics of the sample (N=39) correlation with VEGF-A mRNA expression.

Variables	OSCC with anemia	OSCC non-anemia	VEGF-A Expression (Mean ± SD)	Fold change	p
Total, n (%)	13 (33.3%)	26 (66.6%)	6.84 ± 0.57 5.88 ± 0.44	1.94	0.00*
Tumor size, n (%)				1.44	0.03*
≤ 5 cc	11 (28.2%)	13 (33.3%)	6.87 ± 0.58		
≥ 5 cc	2 (3.8%)	13 (33.3%)	6.34 ± 0.73		
Histological grading				1.21	0.27
Low grade	7 (17.9%)	21 (53.8%)	6.66 ± 0.70		
High grade	6 (15.3%)	5 (12.8%)	6.32 ± 0.69		

Table 3. Severity of anemia (N=13) correlation with VEGF-A mRNA expression

Samples	Anemia		VEGF-A Expression (Mean ± SE)	Fold change	p
	Mild	Moderate			
Number of sample groups I	10 (53.8%)	3 (7.6%)	5.79 ± 0.30	1.30	0.19
Mild anemia					
Female (10,0–11,9 gr/dl)	3 (7.6%)	3 (7.6%)			
Male (10,0–12,9 gr/dl)	2 (3.8%)	2 (3.8%)			
Moderate anemia					
8,0–9,9 gr/dl	0	3 (7.6%)	6.182 ± 0.77		

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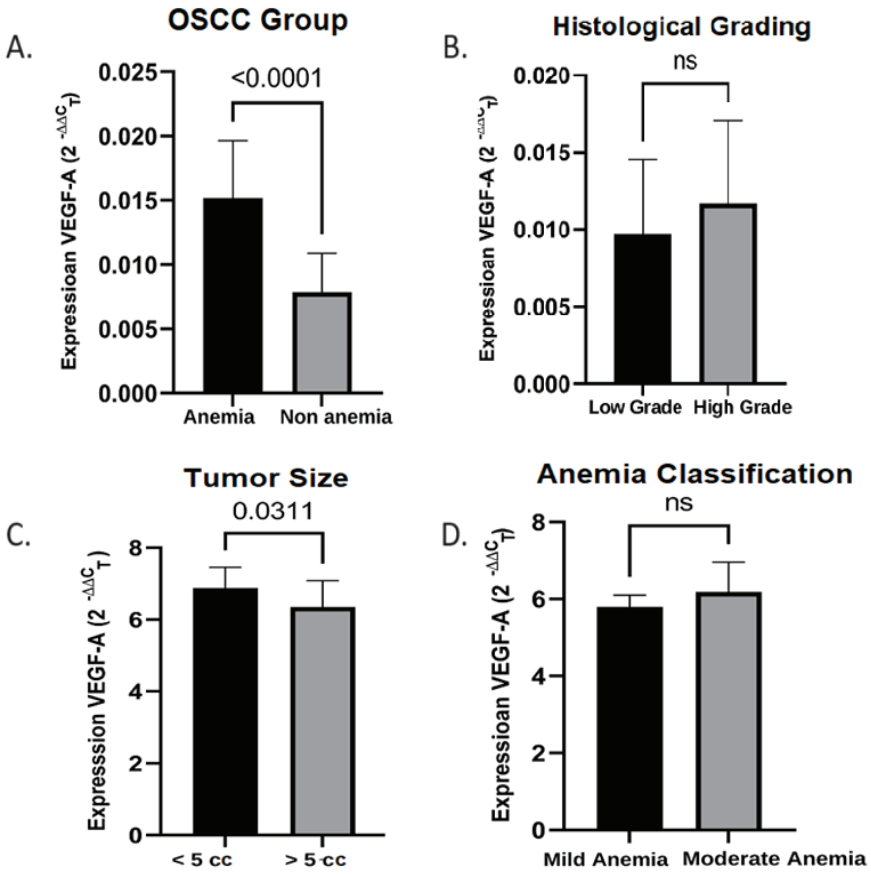

Figure 1. VEGF-A mRNA expression in oral squamous cell carcinoma (OSCC) patients. VEGF-A mRNA expression levels were compared between different patient groups using independent t-tests. (A) Anemic patients (n = 13) had higher expression compared to non-anemic patients (n = 26) (p = 0.00); (B) Patients with larger tumors (> 5 cm, n = 13) exhibited significantly higher expression than those with smaller tumors (≤ 5 cm, n = 26) (p < 0.05); (C) No significant difference was observed between low-grade (n = 22) and high-grade (n = 17) tumors (p > 0.05); (D) Anemia severity (mild n=10, moderate n = 3, non-anemic n = 26) (p > 0.05). Data are presented as mean ± standard deviation. *p < 0.05, ns: not significant.

DISCUSSION

This study demonstrates a significant correlation between VEGF-A mRNA expression and anemia status in oral squamous cell carcinoma (OSCC) patients, as well as a relationship with tumor size. These findings support our hypothesis that anemia and tumor progression in OSCC are associated with increased VEGF-A expression, potentially through hypoxia-induced angiogenesis [18,19].

The observed increase in VEGF-A mRNA expression in anemic OSCC patients aligns with previous studies linking anemia to enhanced tumor angiogenesis. Misra et al. [22] reported that anemia in cancer patients leads to lower oxygen pressure, resulting in increased HIF-1α activity and subsequent VEGF synthesis [19–22]. Similarly, Cordella et al. [23] found that hemoglobin levels below 12 g/dL in cancer patients were associated with increased hypoxia and reduced oxygen supply. Our results extend these findings specifically to OSCC, suggesting that anemia-induced hypoxia may be a key driver of VEGF-A expression in this cancer type.

The correlation between VEGF-A expression and tumor size in our study is consistent with the work of Ueda et al. [25], who reported that increased VEGF-A expression could lead to invasive carcinoma through enhanced microvascular permeability and endothelial cell growth [24,25]. Naderi et al. [9] also demonstrated a relationship between VEGF expression and tumor staging in OSCC, with larger tumors showing higher VEGF levels due to increased oxygen demand [9,20,26]. These findings collectively support the role of VEGF-A in promoting tumor growth through angiogenesis.

Interestingly, we did not observe a significant correlation between VEGF-A expression and histological grading. This unexpected result suggests that other molecular pathways, such as Wnt and β-catenin signaling, may play a more prominent role in determining tumor differentiation in OSCC, as proposed by previous studies.

The implications of our findings are twofold. First, they highlight the potential of VEGF-A as a biomarker for OSCC progression, particularly in the context of anemia. Second, they suggest that addressing anemia in OSCC patients may be crucial for managing tumor growth and improving treatment outcomes. Future research should explore the efficacy of combining anti-angiogenic therapies with treatments targeting anemia in OSCC patients.

Our study has limitations, including its cross-sectional nature, which precludes the establishment of causal relationships. Additionally, we did not investigate the molecular mechanisms underlying the observed associations. Future longitudinal studies and in vitro experiments could provide deeper insights into the causal relationships between anemia, VEGF-A expression, and OSCC progression.

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CONCLUSIONS

This study demonstrates a significant difference in vascular endothelial growth factor-A (VEGF-A) mRNA expression between oral squamous cell carcinoma (OSCC) patients with and without anemia. Specifically, we observed a 1.94-fold increase in VEGF-A mRNA expression in OSCC patients with anemia compared to those without anemia. These findings suggest a potential relationship between anemia and VEGF-A expression in OSCC. These findings contribute to our understanding of OSCC pathogenesis and may inform the development of novel diagnostic and therapeutic strategies.

Further research is needed to explore the clinical implications of this association and to investigate the underlying mechanisms linking anemia to VEGF-A expression in OSCC patients.

DECLARATIONS

Competing interest

The authors declare that they have no competing interests, financial or non-financial, that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Jenderal Soedirman University, approval number: 026/KEPK/PE/V/2022.

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