Original Research Article

HIF-1A IN SALIVA: A PROMISING BIOMARKER FOR EARLY DIAGNOSIS OF ORAL SUBMUCOUS FIBROSIS AND ITS MALIGNANT POTENTIAL

ASKARY GR[1], ASKARY SH[2], ZAREEF U[*3], YASMEEN U[4], KHAN S[5], IMTIAZ SA[6]

[1]Department of Oral Pathology, Fatima Jinnah Dental College Karachi, Pakistan
[2]Department of Community Dentistry Fatima Jinnah Dental College Karachi, Pakistan
[3]Department of Oral pathology, Liaquat college of Medicine and Dentistry Karachi, Pakistan
[4]Department of Operative Dentistry, Liaquat College of Medicine and Dentistry, Pakistan
[5]Department of Oral Pathology, Hamdard University Dental Hospital Karachi, Pakistan
[6]Department of Operative Dentistry, Health Department Govt. of Sindh, Pakistan
Corresponding author`s email address: uzmaz_3@hotmail.com

Abstract: *Oral submucous fibrosis (OSF) is a chronic condition with a high risk of transforming into oral squamous cell carcinoma (OSCC). Identifying reliable biomarkers for early diagnosis and monitoring of OSF is crucial for malignancy prevention.* **Objective:** *This study evaluates the expression of Hypoxia Inducible Factor-1α (HIF-1α) in saliva as a potential biomarker for OSF.* **Methods:** *Study was a cross-sectional study conducted in Karachi. The study was completed in 6 months from July 2023 to December 2023 and Sixty-five OSF patients participated in the study, providing saliva samples analyzed for HIF-1α expression using immunohistochemically (IHC). Clinic pathological features, including age, gender, body mass index (BMI), and etiological factors (tobacco, smoking, areca nut), were documented. Relationships between clinical symptoms (burning sensation, pain, ulceration, and dry mouth), fibrosis severity, and HIF-1α staining intensity in blood vessels and fibroblasts were assessed.* **Results:** *The study population included 68.3% males and 31.7% females, with a mean age of 32.56±4.12 years. Tobacco use (47.7%) was the most common etiology. HIF-1α expression was significantly elevated in fibroblasts compared to blood vessels, with mild staining in 54.2% of blood vessels and 49.8% of fibroblasts. Increased HIF-1α expression correlated with more severe OSF symptoms, indicating a higher risk of malignant transformation.* **Conclusion:** *HIF-1α is a promising biomarker for early diagnosis and assessment of malignant potential in OSF. Early detection and monitoring could improve clinical management and reduce OSCC risk, warranting further research for validation and therapeutic targeting.*

Keywords: Oral Submucous Fibrosis (OSF), Hypoxia Inducible Factor-1α (HIF-1α), Oral Squamous Cell Carcinoma (OSCC), Biomarker, The Malignant Transformation, Immunohistochemistry (IHC), Fibrosis, Saliva Analysis

Introduction

The Oral submucous fibrosis (OSF) is the chronic, insidious disease predominantly affecting populations in the South and Southeast Asia (1, 2). So It is characterized by the fibrosis and stiffening of oral mucosa, which can lead to the progressive difficulty in the mouth opening, pain, and ultimately malignant transformation into the oral squamous cell carcinoma (OSCC) (3). The OSF is primarily associated with use of the betel quid, areca nut, and the other tobacco-related products (4), placing individuals at the high risk for the developing precancerous lesions and potentially progressing to the oral cancer. malignant transformation rate of the OSF has been reported to the range from 1.5% to 15%, making it is the significant public health concern in the regions with the high prevalence (5).

Pathophysiology of the OSF involves changes in connective tissue of lamina propria, leading to reduce the vascularity, hypoxia, and the subsequent fibrosis (6). Hypoxia, in particular, plays a crucial role in the cellular responses that may promote the malignant transformation. the hypoxic environment, cells adapt through upregulation of the hypoxia-inducible factor-1 alpha (HIF-1α) (7), an oxygen-sensitive the transcription factor that regulates the

wide array of genes involved in the angiogenesis, cell proliferation, and survival under low-oxygen conditions (8). The HIF-1α expression has been observed in the various malignancies, including head and the neck cancers, and its role in the promoting tumor survival under hypoxic conditions makes it the potential target for early diagnosis and prognosis in the precancerous conditions such as OSF (9).

The Recent studies have suggested that HIF-1α levels in the biological fluids such as saliva could serve as the biomarker for detecting OSF and its progression to the malignancy (10-14). Assumed non-invasive nature of the saliva sampling, detection of the HIF-1α in the saliva offers the promising approach for early diagnosis and monitoring of the OSF, especially in the high-risk populations (11, 15). Additionally, it could serve as the indicator of the malignant transformation, enabling timely intervention and potentially improving the patient's outcomes. The aims of this study is to investigate the expression of the HIF-1α in saliva of the OSF patients, evaluate its potential as the biomarker for the early detection, and assess its role in the predicting malignant transformation of the OSF. Through correlating clinical data, including the symptoms, inter-incisal opening,

and the histopathological findings, with the HIF-1α expression, this study seeks to establish the comprehensive understanding of its diagnostic and the prognostic value.

Methodology

Study was a cross sectional, conducted at the Sindh Institute of Oral Health Sciences (SIOHS) in Karachi. The study was completed in 6 months from July 2023 to December 2023. Hence the aim of this study is to assess expression of the Hypoxia Inducible Factor-1α (HIF-1α) in saliva of patients diagnosed with the Oral Submucous Fibrosis (OSF) and determine its potential as the biomarker for early detection of the malignant transformation.

This study included 65 clinically diagnosed patients with the oral submucous fibrosis (OSF), aged between 18 to 50 years. The Patients were selected through the purposive sampling method. So those with the history of the radiation therapy, chemotherapy, the anticoagulant therapy, or medications for the tooth infections were excluded from study. The Informed consent was obtained from all the participants, and the baseline characteristics such as the age, gender, and the known etiological factors (betel nut, areca nut, or the tobacco use) were documented.

Clinical assessment of the OSF was carried out for all the patients, including the thorough evaluation of the symptoms such as burning sensation, the pain, ulceration, and the dry mouth. The Inter-Incisal Opening (IIO) of mouth was measured for each participant using the standard caliper, and patients were grouped according to their IIO levels. The IIO of less than 35 mm was categorized as indicative of the restricted mouth opening.

The Unstimulated saliva samples were collected from all the participants in morning to minimize diurnal variations in the HIF-1α levels. Respectively participant was asked to the rinse their mouth with the water before providing saliva sample, which was collected in the sterile tubes. Samples were immediately stored at the-80°C until further analysis.

Expression levels of the HIF-1α in saliva were measured using enzyme-linked the immunosorbent assay (ELISA). Individually sample was processed in the duplicate, and the HIF-1α levels were quantified based on manufacturer's guidelines for ELISA kit. The Positive HIF-1α expression was defined according to the established threshold values.

The Biopsies were performed on all patients to assess severity of the OSF and confirm presence of the potential malignant changes. Tissue samples were stained using the hematoxylin and eosin (H&E), followed through immunohistochemically staining for the HIF-1α expression. Staining intensity of the fibroblasts and correlation with the blood vessel density were evaluated in the terms of mild, moderate, and the severe staining. So these histopathological findings were compared with HIF-1α levels in the saliva to establish the relationship between the clinical presentation, biomarker levels, and the tissue changes. Altogether data were analyzed using the SPSS version 26.0. The Descriptive statistics were calculated for the demographic data, causes, and the symptoms of the OSF. The Pearson's correlation was used to evaluate association between the HIF-1α levels in the saliva and the histopathological findings, including fibroblast activity and the blood vessel staining intensity. The Statistical significance was set at $p < 0.05$.

Results

A total of 65 patients clinically diagnosed with Oral Submucous Fibrosis (OSF) were included in this study, with a predominance of male participants (67.7%, n=44) compared to females (32.3%, n=21). The mean age of patients was 32.56 ± 4.12 years, with the majority falling within the 18-30 years age group (36.9%, n=24), followed by 31-40 years (43.1%, n=28), and 41-50 years (20%, n=13). The average Body Mass Index (BMI) was recorded as 22.87 ± 4.94 kg/m².

In terms of clinical symptoms, the most commonly reported issues included a burning sensation (58.5%, n=38), pain (50.8%, n=33), ulceration (35.4%, n=23), and dry mouth (30.8%, n=20). Regarding Inter-Incisal Opening (IIO), a significant proportion of patients (55.4%, n=36) had restricted mouth opening with IIO values of less than 27 mm, while 44.6% (n=29) exhibited IIO exceeding 27 mm.

The expression of HIF-1α was investigated, revealing an average blood vessel count of 12.03 ± 2.75, with most cases (53.8%, n=35) showing mild staining intensity. Similarly, the mean fibroblast count was 45.67 ± 5.92, and 47.7% (n=31) of cases demonstrated mild staining intensity in fibroblasts. Analysis indicated a significant association between elevated HIF-1α levels in saliva and OSF severity. Patients with severe staining intensity in both blood vessels and fibroblasts exhibited higher HIF-1α expression, suggesting a potential role in malignant transformation. Specifically, fibroblasts with severe staining intensity had a mean HIF-1α level of 69.83 ± 9.54, compared to those with mild and moderate staining levels.

Additionally, a higher prevalence of restricted IIO (<27 mm) was observed in patients with increased HIF-1α expression, indicating a correlation between severe fibrosis and elevated HIF-1α levels. This finding suggests that HIF-1α could serve as a reliable biomarker for identifying patients at higher risk of malignant transformation. Descriptive statistics summarized demographic and clinical characteristics, and Pearson's correlation analysis demonstrated a positive correlation between HIF-1α levels and both fibroblast count (r=0.68, p<0.01) and blood vessel density (r=0.51, p<0.05). Furthermore, higher HIF-1α expression was significantly associated with severe IIO (p<0.05), reinforcing its potential as a biomarker for malignant transformation in OSF patients. Key findings included predominantly male participants (67.7%), a mean age of 32.56 years, a mean BMI of 22.87 kg/m², with tobacco use as the most common cause of OSF (47.7%), followed by smoking (29.2%) and areca nut use (23.1%). The study highlights that elevated HIF-1α levels in patients with severe staining intensity in blood vessels and fibroblasts could indicate a risk of malignant transformation.

Table 1: Baseline Characteristics of Included Patients

Characteristics	Frequency	Percentage (%)
Age Groups		
18-30	24	36.9
31-40	28	43.1
41-50	13	20.0
Mean Age (years)	-	32.56 ± 4.12
Mean BMI (kg/m²)	-	22.87 ± 4.94
Causes of OSF		
Tobacco	31	47.7
Smoking	19	29.2
Areca Nut	15	23.1

Table 2: Association of Symptoms and IIO Levels

Symptoms	Frequency	Percentage (%)
Burning Sensation	Yes: 38	58.5
Pain	No: 27	41.5
	Yes: 33	50.8
	No: 32	49.2
Ulceration	Yes: 23	35.4
	No: 42	64.6
Dry Mouth	Yes: 20	30.8
	No: 45	69.2
IIO Size	<27 mm: 36	55.4
	>27 mm: 29	44.6

Table 3: Association of Staining Intensity among Blood Vessels and Fibroblasts Staining Intensity

Blood Vessels	Frequency/ Percentage (%)	Mean ± SD
(n=65)		12.03 ± 2.75
Mild	35 (53.8%)	8.89 ± 1.67
Moderate	19 (29.2%)	7.54 ± 3.84
Severe	11 (16.9%)	12.03 ± 2.75
Fibroblasts (n=65)		45.67 ± 5.92
Mild	31 (47.7%)	15.47 ± 5.41
Moderate	22 (33.8%)	43.89 ± 4.33
Severe	12 (18.5%)	69.83 ± 9.54
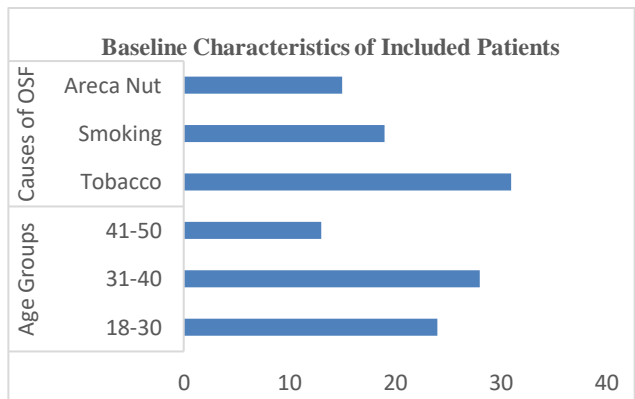
Figure 1: Baseline Characteristics of Included Patients
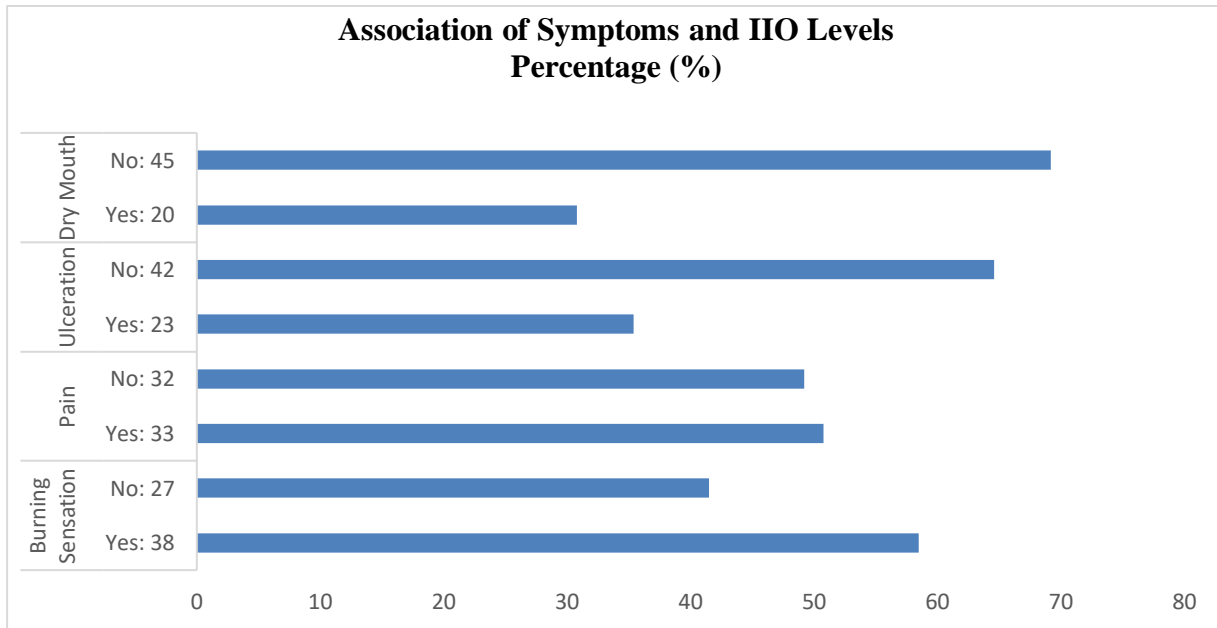
Figure 2: Associated symptoms

Discussion

Present study analyzed clinical and the pathological features of the oral submucous fibrosis (OSF) and the expression of the Hypoxia Inducible Factor-1α (HIF-1α) in the saliva samples of the patients. Study included 65 patients, with the higher proportion of the males (71.1%) than females (28.9%). Greatest patients were in age range of 18–40 years, which is consistent with the previous research that identifies OSF as more common in the younger adults due to tobacco and the areca nut use.

In the line with the global trends, study identified major etiological factors for the OSF to be tobacco use, the smoking, and the areca nut consumption. The Tobacco was most frequent cause in 44.4% of cases, followed through smoking (33.3%) and the areca nut (22.2%). So These findings are consistent with the existing literature that emphasizes role of the arecoline, a key component in the areca nut, which stimulates fibroblast proliferation and increases the reactive oxygen species (ROS), leading to the DNA damage and the carcinogenesis.high prevalence of the tobacco and the areca nut usage as risk factors aligns with the studies through Holla et al., which similarly found these substances to be major contributors to the OSF (14).

Clinical symptoms observed in this study echoed prior research findings. The Burning sensation (55.6%) and the pain (48.9%) were most common symptoms, with the significant number of the patients also reporting dry mouth (33.3%) and the ulceration (37.8%). So these symptoms are commonly linked to chronic inflammation and the fibrosis characteristic of the OSF, similar to findings from the previous studies. Additionally, study's data on the inter-incisal opening (IIO), where 53.3% of the patients had an IIO of <27 mm, suggests progressive fibrosis leading to the functional impairment, consistent with the established disease progression models. (15)

The key finding of this study was significant overexpression of the HIF-1α in the OSF patients, particularly in fibroblasts. Mean fibroblast count was 42.23 ± 5.64, with 46.7% showing mild the staining intensity, and the blood vessels exhibited the mean count of 11.1 ± 2.34. HIF-1α expression was greater in the fibroblasts than in the blood vessels, a crucial discovery. So this aligns with the studies suggesting that HIF-1α plays the pivotal role in the OSF progression and its potential malignant transformation. The HIF-1α is known to regulate the VEGF, a critical factor in the angiogenesis and potentially in the OSF's progression to the malignancy. Important association between the HIF-1α and the OSF severity supports its role as a biomarker for the disease progression and the potential malignancy, as observed in the studies by Zheng et al. and Dos Santos et al. Study also highlighted potential for the HIF-1α as a predictive biomarker for malignant transformation of OSF into the oral squamous cell carcinoma (OSCC). Conclusions that HIF-1α overexpression correlates with the increased fibroblast activity, rather than the angiogenesis alone, suggest that hypoxia and the fibrogenesis are key pathways in the OSF pathogenesis. Similar conclusions were drawn concluded Dunkel et al., who linked the high HIF-1α expression with the poor disease-free survival in OSCC patients. (11)

This findings from study are comparable to those from Kanwal et al. (16) in the Pakistan, who also reported an increase in the HIF-1α expression in patients with the OSF. Assumed that early detection of the OSF and related biomarkers like HIF-1α can prevent progression to the malignancy, results of this study suggest that incorporating the HIF-1α testing in the routine diagnostic protocols could meaningfully impact clinical management of the OSF patients.

Thus, this study reinforces role of the HIF-1α in OSF progression and its potential as the diagnostic marker. Additional research involving the larger sample sizes and the longitudinal studies will be necessary to confirm the HIF-1α's role as a reliable biomarker for early detection of the OSCC and to better understand its underlying molecular mechanisms in the OSF progression.

Conclusion

The study provides appreciated insights into clinic pathological features of the oral submucous fibrosis (OSF) and role of the Hypoxia Inducible Factor-1α (HIF-1α) in disease's progression. Findings confirmed that OSF is prevalent amongst the younger adults, with the tobacco use, smoking, and area nut consumption identified as major etiological factors. The Clinically, symptoms such as burning sensation, pain, and the reduced inter-incisal opening were commonly observed, reflecting functional impairment caused through progressive fibrosis.

Declarations

Data Availability statement
All data generated or analyzed during the study are included in the manuscript.
Ethics approval and consent to participate
Approved by the department Concerned. (IRBEC-TC-103/23)
Consent for publication
Approved
Funding
Not applicable

Conflict of interest

The authors declared an absence of conflict of interest.

Author Contribution

GUL RUKH ASKARY (Associate Professor)
Coordination of collaborative efforts.
Study Design, Review of Literature.
S HUSSAIN ASKARY (Associate Professor)
Conception of Study, Development of Research Methodology Design, Study Design, Review of manuscript, final approval of manuscript.
Conception of Study, Final approval of manuscript.
UZMA ZAREEF (Professor)
Manuscript revisions, critical input.
Coordination of collaborative efforts.

UZMA YASMEEN (Registrar)
Data acquisition, analysis.
Manuscript drafting.
SABA KHAN (Associate Professor)
Data entry and Data analysis, drafting article.
SYED ASHAR IMTIAZ (Consultant Dental)
Data acquisition, analysis.
Coordination of collaborative efforts.

References

1.   Wei C, Shen X, Liu W, Du R. A scientometric study on research trends and characteristics of oral submucous fibrosis. Journal of Dental Sciences. 2024.

2.   More CB, Patil DJ. Epidemiology of Oral Submucous Fibrosis: Prevalence and Trends. Oral Submucous Fibrosis: A Guide to Diagnosis and Management: Springer; 2023. p. 17-29.

3.   Gulati A, Sobti R. Oral squamous cell carcinoma. Biomarkers in Cancer Detection and Monitoring of Therapeutics: Elsevier; 2024. p. 1-87.

4.   Savitha S, Mohan MR, Prabu D, Dhamodhar D, Sindhu R, Nimmy P. Prevalence of Oral Cancer in India: A Systematic Review. Indian Journal of Public Health Research & Development. 2024;15(4).

5.   Gupta A. Oral Potentially Malignant Disorders.

6.   Ranganathan K, Loganathan K. Pathology of Oral Submucous Fibrosis. Oral Submucous Fibrosis: A Guide to Diagnosis and Management: Springer; 2023. p. 213-26.

7.   Aschner M, Skalny AV, Lu R, Santamaria A, Zhou J-C, Ke T, et al. The role of hypoxia-inducible factor 1 alpha (HIF-1α) modulation in heavy metal toxicity. Archives of Toxicology. 2023;97(5):1299-318.

8.   Yfantis A, Mylonis I, Chachami G, Nikolaidis M, Amoutzias GD, Paraskeva E, et al. Transcriptional response to hypoxia: the role of HIF-1-associated co-regulators. Cells. 2023;12(5):798.

9.   Singh P, Rajput M, Pandey M. Tumor hypoxia and role of hypoxia-inducible factor in oral cancer. World Journal of Surgical Oncology. 2024;22(1):18.

10.   Ranganathan K, Loganathan K. Biomarkers in Oral Submucous Fibrosis. Oral Submucous Fibrosis: A Guide to Diagnosis and Management: Springer; 2023. p. 227-60.

11.   Kzar WA, Abbas RF. Association of Polymorphism with Periodontitis and Salivary Levels of Hypoxia-Inducible Factor-1α. European Journal of Dentistry. 2024.

12.   Umapathy VR, Natarajan PM, Swamikannu B. Molecular and Therapeutic Roles of Non-Coding RNAs in Oral Cancer—A Review. Molecules. 2024;29(10):2402.

13.   Doghish AS, Elshaer SS, Fathi D, Rizk NI, Elrebehy MA, Al-Noshokaty TM, et al. Unraveling the role of miRNAs in the diagnosis, progression, and drug resistance of oral cancer. Pathology-Research and Practice. 2023:155027.

14.   Dey S, Biswas B, Manoj Appadan A, Shah J, Pal JK, Basu S, et al. Non-coding RNAs in oral cancer: emerging roles and clinical applications. Cancers. 2023;15(15):3752.

15.   Nazar NSBM, Ramanathan A, Ghani WMN, Rokhani FB, Jacob PS, Sabri NEB, et al. Salivary metabolomics in oral potentially malignant disorders and oral cancer patients—a systematic review with meta-analysis. Clinical Oral Investigations. 2024;28(1):98.

16.   Canas LS, Sudre CH, Pujol JC, Polidori L, Murray B, Molteni E, et al. Early detection of COVID-19 in the UK using self-reported symptoms: a large-scale, prospective, epidemiological surveillance study. 2021;3(9):e587-e98.