Salivary Levels of Hypoxia Inducible Factor-1α in Stage III Oral Submucous Fibrosis: A Comparative Clinical and Laboratory Study

Kanwal Iqbal[1*] and Saima Butt[1]

[1]*Department of Oral Pathology, Ziauddin University, Karachi, Pakistan*

Abstract

Background: A persistent, crippling disease known as oral submucous fibrosis (OSMF) is distinguished by fibrosis of the juxta-epithelium. It has been proposed that betel quid chewing and its malignant transformation of OSMF are associated with hypoxia, a major micro-environmental component in OSMF. The primary regulator of hypoxia in cellular response, HIF-1α is significantly up-regulated in several fibrotic disorders, including OSMF.

Objective: The objective of this study was to examine the expression of the protein HIF-1α in the salivary samples of healthy controls and stage III OSMF.

Methods: It was a case-control study carried out in the Department of Oral Medicine, Ziauddin University, Karachi from July 2020 to May 2021. There were 57 participants in the present study, 29 healthy controls and 28 patients with stage III OSMF. The method of non-probability consecutive sampling was employed. Demographic information was gathered using a standardized baseline questionnaire. The enzyme-linked immunosorbent assay was used to measure the amount of HIF-1α present in saliva. To find out if the data was distributed normally, the Shapiro-Wilk test was performed. To compare the expression of HIF-1α protein between the two study groups, the Mann-Whitney test was used.

Results: In the stage III OSMF group, the mean salivary HIF-1α value was 25.64±1.27 ng/ml, while in the control group, it was 12.30 ± 1.58 ng/ml. The Mann-Whitney U-test revealed a statistically significant difference ($p< 0.001$). A significant negative correlation ($r = -0.526$, $p = 0.000$) was seen in the individual group analysis between salivary HIF-1α and mouth opening.

Conclusion: In comparison to the healthy individuals, the stage III OSMF group had higher levels of HIF-1α protein. The results of our investigation suggest that HIF-1α could be a valuable marker of malignant transformation in OSMF.

Keywords: *Oral submucous fibrosis, saliva, HIF-1α, overexpression, malignant transformation.*

INTRODUCTION

The malignant transformation rate for oral submucous fibrosis ranges from 7-30%, making it a high-risk precancerous disease. Around 5 million individuals globally, primarily in southern India, are affected by OSMF [1]. The deeper connective tissue of the oral mucosa and fibrosis of lamina propria are characteristics of OSMF [2]. Numerous studies have shown that the consumption of areca nut in different formulations is a substantial risk factor for OSMF. Due to the widespread usage of smokeless chewable tobacco and areca nuts in Southeast Asia, Pakistan has a higher incidence of OSMF due to the products' easy accessibility [3]. The incidence of OSMF was found to be greater (99%) in users of areca nut and associated products in a study done in a rural region of Sindh, Pakistan [4]. Furthermore, research on teenagers has shown an association between areca nut consumption and OSMF, with 50–79.6% of users developing OSMF [5].

An essential role in tumor angiogenesis is played by HIF-1α, an oxygen-dependent transcriptional activator [6]. The intensity and function of HIF-1α are regulated by other post-translational changes, hydroxylation, acetylation, and phosphorylation. The protein factors such as prolyl hydroxylase (PHD), von Hippel-Lindau tumor suppressor gene (pVHL), and E1A-associated protein/ CREB-binding protein (p300/CBP) are related to HIF-1α [7]. Under normoxic conditions, the ubiquitin-proteasome pathway driven by pVHL leads to the rapid degeneration of HIF-1α. Within the polypeptides, oxygen-dependent degradation (ODD) domain region, lysine, and proline are hydroxylated and acetylated, respectively, causing pVHL and HIF-1α to associate in normoxic conditions. In contrast, the hypoxic state makes the HIF-1α component immobile, and it binds to protein co-activators like p300/CBP to control its transcriptional activity. Ultimately, in a hypoxic environment, HIF-1α functions as the primary regulator of several hypoxia-inducible genes [8].

There is evidence that chewing betel quid and its malignant transformation are associated with hypoxia, a major micro-environmental component in OSMF. The primary regulator of hypoxia in cellular response, HIF-1α is significantly up-regulated in several fibrotic disorders, including OSMF [9]. A correlation between hypoxia and fibrosis in renal and lung fibroblasts has also been demonstrated in the literature [10].

Based on the aforementioned data, we postulated that HIF-1α may be a biomarker for early OSMF identification

**Corresponding author: Kanwal Iqbal, Department of Oral Pathology, Ziauddin University, Karachi, Pakistan, Email: kanwal.iqbal@zu.edu.pk*
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and may be able to stop the precancerous condition from developing into cancer in the highly affected population. To determine the differences in HIF-1α protein levels between saliva samples from patients with stage III OSMF and healthy controls, a clinical and laboratory investigation was conducted.

MATERIALS AND METHODS

Study Design and Duration

It was a case-control study. The participants were recruited from the Department of Oral Medicine, Ziauddin University, Karachi. The duration of the study was from July 2020 to May 2021.

Ethical Considerations

The Ziauddin University Ethics Research Committee reviewed this work (Reference code: 1840120KIOM). Before the protocol started, all enrolled patients were allowed to give informed consent.

Power Calculation

The sample size was calculated from the mean difference of HIF-1α between two groups [11], 80% power at a confidence level of 95% through www.openepi.com using a case-control study design scientific calculator. The initial sample size calculated was 8, but to increase the significance of our study, we included a total of 57 participants (28 cases and 29 controls) using non-probability consecutive sampling.

Study Patients and Selection Criteria

There were 57 participants in total for this study, divided into two groups: 29 individuals who were systemically and orally healthy and 28 people who had stage III OSMF. Stage III OSMF was selected because most patients who came to OPD were of stage III. The test group included people who had a history of using chewable tobacco and areca nuts without apparent preference for one gender. OSMF patients with any chronic oral or systemic ailment or those who had received prior steroid treatment were excluded.

Research Questionnaire

A professional examiner gave each participant the baseline structured questionnaire sheet. Details including age, gender, occupation, ethnicity, and habits (such as using gutka, areca nut, and betel quid) as well as their duration and frequency were requested in the questionnaire.

Clinical Examinations

Every participant underwent a clinical examination. All OSMF patients were diagnosed following the clinical criteria put forth by Chandramani *et al.*, using the stage III OSMF intraoral assessment: a) stomatitis; b) presence of palpable fibrous bands, and c) inter-incisal mouth opening [12].

Saliva Collection and Estimation of Salivary HIF-1α

Every individual underwent the passive drool technique to obtain unstimulated whole saliva (UWS) samples. Each participant had five milliliters (ml) of UWS sampled in sterile falcon tubes following the clinical evaluation and interview. For five minutes, the participants were told to gather saliva in their mouths while seated comfortably with their heads slightly bowed. After collecting their saliva, it was in a large funnel that was attached to a falcon tube that was inserted into a cup of dry ice and taken straight to the lab. The samples were placed in several aliquots and centrifuged at 1792 x g for 10 min at 4°C before being further examined at -80°C. An enzyme-linked immunosorbent assay (ELISA) (SEA798Hu, USCN, USA) was used to determine the levels of HIF-1α present in saliva.

Statistical Analysis

Specialised statistics software (SPSS version 23) was used for all data analysis. The data about demographics and salivary HIF-1α levels were presented as means with standard deviations. The Shapiro-Wilk test was used to determine whether each variable had a normal distribution. The Mann-Whitney test was used to compare the levels of HIF-1α between the test and the control groups. The Spearman correlation test was used to examine correlations between the independent variables and the salivary HIF-1α level. p-value <0.05 was considered statistically significant.

RESULTS

General Features of Research Participants

There were 57 participants in the study: 29 healthy controls and 28 people with stage III OSMF. Participants in the test group comprised 60.7% of men, while those in the control group it was 37.9%. The mean age of the OSMF group was 36.92 years, while the healthy control group was 31 years. Among the 28 stage III OSMF patients, 16 used betel nut (12.25 packets per day), 6 used betel nut and pan (8 packets/day), 2 used gutka (4 packets/day), 2 used pan and gutka (6 packets/day), 1 used pan only (4 packets/day) and 1 used betel nut and gutka (5 packets/day). The mean duration of habit use among stage III OSMF patients was 12.54 years. In patients with stage III OSMF, the mean mouth opening was 42.41 mm, while in healthy controls it was 21.26 mm.

Of the patients with OSMF, 22 patients showed bilateral buccal mucosal fibrosis, 4 had unilateral buccal mucosal fibrotic bands, 11 reported tongue mobility restriction, and 2 had mild soft palate fibrosis. 12 patients did not experience any burning sensation in their mouth, compared to the total of 16 patients who experienced it.

Table **1** displays the mean salivary HIF-1α levels for patients with stage III OSMF and healthy controls. The mean value of salivary HIF-1α levels was 25.64±1.27 ng/ml and 12.30 ± 1.58 ng/ml for the OSMF group and healthy controls respectively. The OSMF group and the control group had statistically significant differences in HIF-1α levels (p <0.001), as indicated by the Mann-Whitney U-test. (**Fig. 1**).

Table 1: Mean salivary HIF-1α levels in stage III OSMF patients and healthy controls.

Variable	Group (n)	Mean ± SD (ng/ml)	Median	IQR	Lower bound	Upper bound	p-value
Salivary HIF-1α levels	Stage III OSMF (28)	25.64±1.27	27.55	10.72	23.03	28.25	0.000*
	Healthy controls (29)	12.30±1.58	10.01	15.90	9.05	15.56	

*p-value<0.05 was considered significant; Mann Whitney test was applied.

Table 2: Spearman Correlation Analysis among salivary HIF-1α, Habits and Mouth opening.

Variables	Cases (n=28)	
	Correlation Coefficient	p-Value
Duration of betel nut	-0.110	0.686
Frequency of betel nut	-0.044	0.871
Duration of betel nut and pan	-0.205	0.741
Frequency of betel nut and pan	-0.316	0.604
Mouth opening	-0.526	0.000

*p-value<0.05 was considered significant; Spearman correlation was applied.

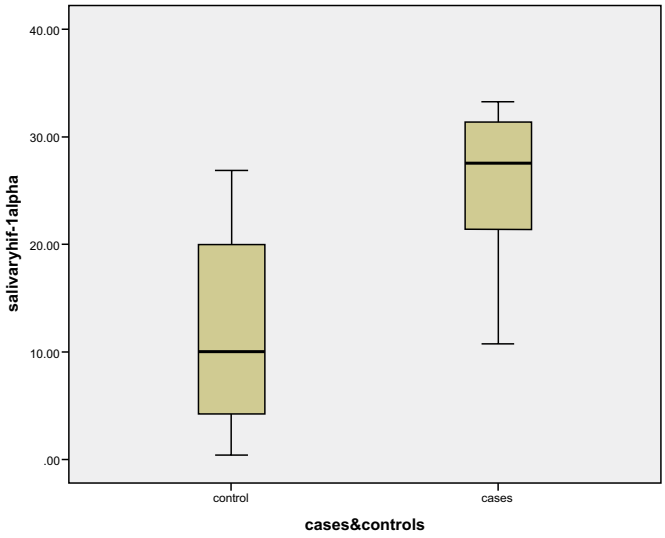

Fig. (1): The Box and Whisker plot displays the mean salivary HIF-1α levels for both healthy controls and OSMF stage III patients.

Spearman Correlation Showed a Comparison of Salivary HIF-1α Levels and Clinical Variables

The correlations between salivary HIF-1α levels and the various clinical variables evaluated in individuals with stage III OSMF were assessed using the Spearman correlation coefficient. The salivary HIF-1α levels did not significantly correlate with the habit's frequency or duration. Furthermore, a significant negative correlation was seen between the salivary HIF-1α and mouth opening (r = -0.526, p = 0.000) (**Table 2**).

DISCUSSION

The salivary HIF-1α levels were hypothesized to be greater in individuals with stage III OSMF than in healthy controls in this investigation, and a correlation between these levels and other clinical variables was discovered. According to our findings, people with stage III OSMF exhibited significantly more HIF-1α than did healthy controls. HIF-1α levels in saliva were found to be significantly negatively correlated with mouth opening, meaning that an increase in levels would follow a decrease in mouth opening.

A well-known phenomenon is tumor hypoxia, which is caused by an imbalance between the availability and use of oxygen inside the tumor microenvironment. Long-term hypoxia can induce certain tumor cells to adapt to biological alterations. This can result in a more aggressive phenotype that can lead to invasion and metastasis [13]. In response to hypoxia, fibroblasts and epithelial cells in renal and lung tissue express HIF-1α more frequently [14, 15]. The connective tissue of OSMF can be hypothesized in the same way. Increased expression of HIF-1α is the outcome of tissue hypoxia caused by reduced vascularity caused by extreme connective tissue fibrosis [16]. According to our research, patients with stage III OSMF had greater HIF-1α levels than healthy controls. Thus, our results imply that HIF-1α might be a key biomarker of malignant transformation in OSMF.

There are certain molecular distinctions between early and late stages of cancer as well as normal, oral, and potentially malignant diseases. The biomarkers have been developed recently using already-established biology procedures; possible biomarkers for OSMF include protein and trace elements from serum and saliva in a solid and liquid biopsy. The salivary levels of copper, LDH, MDA, and S100A7 in OSMF patients were measured in various studies [17-20]. The saliva samples are particularly advantageous when used for analysis. Saliva contains a large amount of proteins and genetic material, is non-invasive, and is simple to collect [21]. Even though histology is the most reliable examination method, it is invasive, has a lengthy wait for test results, and is not well-liked by the patients. Body fluid biopsy testing has recently been shown to be less invasive, have shorter waiting times for test results, and have a high level of patient acceptance; however, additional supporting data are required to prove accuracy. To prove the accuracy of most of the novel biomarkers, a large sample size is required. Currently, OSMF diagnosis still relies heavily on clinical signs and symptoms and pathological investigation. When evaluating different proteins routinely in clinical trials, saliva is the preferred fluid [22].

There are certain drawbacks in this study. Since the investigation is cross-sectional, the characteristics of the data prevent us from comprehending the pathophysiology of OSMF and its molecular association with salivary HIF-1α levels, which were assessed concurrently with clinical tests. Furthermore, to determine the extent of fibrosis, we

did not perform microscopic evaluations. Consequently, by analyzing HIF-1α levels and correlating them with soft tissue changes in OSMF, longitudinal studies can be helpful.

It is possible that measuring HIF-1α levels in saliva could be just as sensitive and precise as measuring levels in serum to differentiate between precancerous and healthy states, as our work demonstrates that stage III OSMF patients express higher levels of HIF-1α in their saliva than the healthy controls. However, to validate our findings and determine whether saliva is a valid diagnostic fluid, more research should be done and compare salivary HIF-1α levels to those of blood and tissue samples. This research indicates that HIF-1α levels may serve as a diagnostic salivary protein for identifying and categorizing patients who are susceptible to OSMF. This research did not assess the sensitivity or specificity of HIF-1α in saliva. Thus, the cutoff point for HIF-1α diagnostic values in OSMF is yet unknown. The present study covers preliminary data; additional research is necessary to determine threshold levels of HIF-1α in OSMF.

CONCLUSION

The results of this study indicate that salivary HIF-1α levels were higher in stage III OSMF patients than in healthy individuals, indicating a potential function for HIF-1α in the malignant progression of OSMF.

ETHICAL APPROVAL

Ethical approval was obtained from the Ethics Review Committee (ERC) of Ziauddin University, Karachi (REF letter No. 1840120KIOM). All procedures performed in studies involving human participants were following the ethical standards of the institutional and/ or national research committee and with the Helsinki Declaration.

CONSENT FOR PUBLICATION

Written informed consent was taken from the participants.

AVAILABILITY OF DATA

The data set may be acquired from the corresponding author upon a reasonable request.

FUNDING

Declared none.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTION

KI did the conceptualization of the study, literature search, data collection, and writing the article. SB did the proofreading and overall evaluation.

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