Oral Submucous Fibrosis Patients Expressed Hypoxia Inducible Factor-1α (HIF-1α) in Saliva: A Biomarker for Detection of Malignant Transformation

NIGHAT SHAIFIQ1, SADIA AKRAM2, TAHIR ALI KHAN3, NEELOFAR NAUSHEEN4, MUHAMMAD AMER KHAN5, UMAR NASIR6

1Associate Professor Department of Oral Biology, Khyber College of Dentistry, Peshawar, Khyber Pakhtunkhwa
2Assistant Professor Science of Dental Materials, Abbottabad Institute of Medical and Dental sciences, Abbottabad
3Head of Department Dental Materials, Sardar Begum Dental College, Gandhara University, Peshawar
4Assistant Professor, Oral Biology, Sardar Begum Dental College, Gandhara University, Peshawar
5Demonstrator, KMU-Institute of Dental Sciences, Kohat
6Assistant Professor, KMU- Institute of Dental Sciences, Kohat

Corresponding author: Nighat Shafiq, Email: nighatbds@gmail.com

ABSTRACT

Objective: Aim of this current study was to determine the malignant transformation associated with hypoxia inducible factor-1α among patients of oral submucous fibrosis (OSF).

Study Design: This Retrospective study was carried from 1st January, 2021 to 31st December, 2021 at the Department of Dental Materials, Sardar Begum Dental College, Gandhara University, Peshawar.

Methods: There were forty five diagnosed patients of oral submucous fibrosis with ages 18-50 years were included in the current study. After taking informed consent for baseline details were recorded which includes age, gender, causes and symptoms of OSF. Inter Incisal Opening (IIO) of mouth among all the patients were measured. Hypoxia inducible factor-1α was observed among all the cases. Staining intensity of fibroblast and correlation of different grades of staining intensity with blood vessels were assessed in terms of mild, moderate and severe. SPSS 26.0 version was used to analyze complete data.

Results: There were 32 (71.1%) males and 13 (28.9%) females in this study. 22.13±5.29 kg/m² was the mean body mass index of patients with mean age 31.02±3.45 years. Most common cause was tobacco found in 20 (44.4%) cases, followed by smoking in 15 (33.3%) patients and areca nut found in 10 (22.2%) cases. Most common symptom was burning sensation, pain, ulceration and dry mouth. Twenty four (53.3%) patients had IIO level <27mm and >27mm level was in 21 (46.7%) patients. Mean blood vessels were 11.1±2.34 with majority of cases had mild staining intensity in 23 (51.1%) cases. Mean fibroblasts were 42.23±5.64 and had mild staining intensity in majority cases 21 (46.7%).

Conclusion: According to our study HIF-1α was an effective factor in the malignant transformation of oral submucous fibrosis. Aside from that, our findings revealed that OSF malignancy can be detected by the use of HIF-1 as a biomarker.

Keywords: OSF, Hypoxia Inducible Factor-1α, Fibroblast, Staining Intensity, Blood Vessels

INTRODUCTION

OSF is a long-term oral condition that causes scarring and fibrosis of the tissues. Oral cancer is a possible side effect of this condition. As a result, this illness has a high incidence of malignant transformation (1.5–15 percent) [3]. OSF incidence varies by ethnicity and area and is intimately linked to dietary habits, culture, and other lifestyle factors [4–6]. OSF patients are more prevalent in South and South-East Asia [5-8]. OSF’s malignant transformation rate may vary because of the diverse ages, sexes, monitoring periods, risk factors, and pathological diagnoses in the research. Studies have shown that people with OSF have an increased risk of oral cancer. According to previous research, oral cancer development is closely correlated with the length of OSF and the severity of symptoms.[7]

To put it another way, OSF is characterized by changes to the lamina propria’s connective tissue fibres, which in turn leads to a reduction of the oral mucosa's vascularity and subsequently to hypoxia. [9] OSCC is the principal route for malignant transformation from a potentially malignant mucosal transition. Cells and tissues respond to hypoxia by activating a set of genes involved in angiogenesis, iron metabolism, glucose metabolism, cell proliferation, and survival. Hypoxia inducible factor-1α (HIF-1α), an oxygen-sensitive transcriptional activator, is crucial in this process.HIF-1α enters the nucleus where it interacts with hypoxia-responsive transcription factors after stabilising itself in a hypoxic environment.[2] A key regulator of gene expression during hypoxia is HIF-1α. Many growth factors have been related to OSCC in recent research that suggests HIF-1α has a role in the overexpression of VEGF, TGF-b, FGF and platelet-derived growth factor (PDGF) (EGFR). Hypoxia has also been associated to renal and pulmonary fibrosis in fibroblasts.[10]

Oral and pharyngeal cancers are responsible for a large percentage of cancer deaths globally.[6,7] OSCC is more likely to occur on the Indian subcontinent if one consumes tobacco, betel quid, and areca nut. OPC sufferers' survival rates haven't improved in the last three decades.[11]

Most researchers agree that oral cancer manifests itself in phases, beginning with a benign lesion and escalating to malignancy, OSCC has been associated to or preceded by leukoplakia, a precancerous lesion. [12] If dysplasia is severe, malignant transformation can occur as
often as 33 percent of the time, researchers found. One of the most common characteristics of many malignancies is hypoxia. This condition leads directly to the formation of both local and systemic cancers. A lack of appropriate vascularization and blood flow causes an oxygen consumption-to-supply ratio to be out of whack in solid tumours like head and neck squamous cell carcinoma (HNSCC).

Osseo integrative disease (OSD) is the term used to describe the disorder in which the sub mucosal tissues of the mouth develop a chronic, inflammatory, and precancerous fibrosis known as oral submucous fibrosis (OSF). At various phases of the illness, the molecules implicated in the fibrotic process’ biochemical pathways appear to be either downregulated or upregulated.[34]

Cancer cells, on the other hand, adapt to the hostile environment. Keeping solid tumours from becoming hypoxic is essential for the growth and development of the tumour. Cancer cells benefit from hypoxia-protection provided by the hypoxia-inducible factor-1 (HIF-1). When the HIF-1 subunits (alpha and beta) form a heterodimeric HIF-1 protein, it has proteolytic activity. [14] Anticancer therapy is thwarted by hypoxia in human solid tumours, which provides an ideal environment for aggressive cancer cells to grow and thrive. [15] HIF-1 expression has been found in a variety of cancers, including those of the kidney, bladder, colorectal, breast, endometrial, cervical, and head and neck. Due of OSF’s fibrous nature, it is probable that the ill tissues in OSF are in a hypoxic environment. HIF-1 appears to have a function in the cell’s response to hypoxia.

The goal of this study is to see if hypoxia inducible factor-1 is linked to malignant transformation in patients with oral submucous fibrosis.

MATERIAL AND METHODS
This retrospective study was carried out at the Department of Dental Materials, Sardar Begum Dental College, Gandhara University, Peshawar from 1st January, 2021 to 31st December, 2021. Patients were enrolled after taking informed written consent for baseline details included age, gender, causes and symptoms of OSF. Patients had history of radiation therapy, chemo therapy, anticoagulant therapy, use of medicines for tooth infection and <18 years of age were not included in this study.

Forty Five (45) patients with ages 18-50 years were included. Two 3 m-thick tissue slices were cut from the paraffin blocks of the research participants using a semi- automatic microtome. Hematoxylin and eosin were used to stain one region (H & E). For the H&E stained sections, it was also histopathologically assessed using the Pindborg and Sirsat grading process.[19]

On APES-coated slides, the remaining 3 m of tissue was cut into slices and incubated overnight at room temperature (Sigma-Aldrich Chemical Co., USA). Deparaffinization with fresh xylene three times and dehydration three times with pure alcohol needed 15 minutes of heat. An anti-peroxide blocker (Peroxide Blocker) was used for 15 minutes at room temperature, and citrate buffer (pH 6.0) was used for 10 minutes to rinse the peroxides twice. The BioGenex antigen retrieval technology was utilized for antigen retrieval. After being soaked in a citrate buffer solution, the sections were placed in the BioGenex antigen retrieval device and heated for 15 minutes. The slides were cleaned with distilled water for five minutes after the system had cooled to room temperature under running water. The slices were incubated for 15 minutes with a blocking agent to remove the endogenous biotin. Before the sections were incubated for an hour with HIF-1 monoclonal antibody and properly washed with citrate buffer, the extra power block solution had to be removed. Secondary antibody (super-enhancer) was applied for 30 minutes, followed by two 5-minute buffer washes, to enhance the staining on the sections. With the use of horseradish peroxidase, slices were incubated for 30 minutes (BioGenex Life Sciences Pvt., Ltd.). The chromogen diaminobenzidine solution was poured over the sections and well mixed after being combined with one milliliter of buffer. When Harris hematoxylin was counterstained with water and xylene, the operation was finished in 5 minutes.[19]

HIF-1 was previously associated with fibroblasts and blood vessels. For the purposes of fibroblast and blood vessel counting, a Leica research microscope with an integrated camera was utilized. Low-power screening (10) of the stained sections was used to detect areas with increasing staining intensity (Hot spot method). Fibroblasts and blood vessels were counted using a high power (40) magnification microscope. Five of the most densely packed high-power fields (HPFs) were chosen for further study. Each slide’s sample segments were methodically scanned from left to right in order to minimize redundancy. Each HPF was averaged and expressed as either fibroblasts or blood vessels based on the number of cells in that HPF. In order to calculate the mean, ten separate values were averaged together and expressed numerically (standard deviation). In order to eliminate subjective bias, both qualified observers evaluated all immunohistochemistry (IHC)-stained slides and the accompanying H&E slides.

Staining intensity of fibroblast and correlation of different grades of staining intensity with blood vessels were assessed in terms of mild, moderate and severe. SPSS 26.0 version was used to analyze complete data.

RESULTS
There were 32 (71.1%) males and 13 (28.9%) females in this study. Fig 1

![Gender](image_url)

**Figure 1:** Distribution of gender among all the case
22.13±5.29 kg/m² was the mean body mass index of patients with mean age 31.02±3.45 years. Most common cause was tobacco found in 20 (44.4%) cases, followed by smoking in 15 (33.3%) patients and areca nut found in 10 (22.2%) cases. (Table 1)

### Table 1: Included patients with baseline characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
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<tbody>
<tr>
<td>Age groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-30</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>31-40</td>
<td>19</td>
<td>42.2</td>
</tr>
<tr>
<td>41-50</td>
<td>8</td>
<td>17.8</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>31.02±3.45</td>
<td></td>
</tr>
<tr>
<td>Mean BMI (kg/m²)</td>
<td>22.13±5.29</td>
<td></td>
</tr>
<tr>
<td>Causes of OSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td>20</td>
<td>44.4</td>
</tr>
<tr>
<td>Areca Nut</td>
<td>15</td>
<td>33.3</td>
</tr>
<tr>
<td>Smoking</td>
<td>11</td>
<td>24.2</td>
</tr>
<tr>
<td>Mean blood vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;27mm</td>
<td>23</td>
<td>51.1</td>
</tr>
<tr>
<td>&gt;27mm</td>
<td>11</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Most common symptom was burning sensation, pain, ulceration and dry mouth. 24 (53.3%) patients had IIO level <27mm and >27mm level was in 21 (46.7%) patients. (Table 2)

### Table 2: Association of symptoms and level of IIO

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burning Sensation</td>
<td>25</td>
<td>55.6</td>
</tr>
<tr>
<td>Pain</td>
<td>22</td>
<td>48.9</td>
</tr>
<tr>
<td>Ulceration</td>
<td>17</td>
<td>37.8</td>
</tr>
<tr>
<td>Dry Mouth</td>
<td>28</td>
<td>62.2</td>
</tr>
<tr>
<td>IIO Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;27mm</td>
<td>24</td>
<td>53.3</td>
</tr>
<tr>
<td>&gt;27mm</td>
<td>21</td>
<td>46.7</td>
</tr>
</tbody>
</table>

Mean blood vessels were 11.1±2.34 with majority of the cases had mild staining intensity in 23 (51.1%) cases. Mean fibroblasts were 42.23±5.64 and had mild staining intensity in majority cases 21 (46.7%). (Figure 2) (Table 3)

### Table 3: Association of staining intensity among blood vessels and fibroblasts

<table>
<thead>
<tr>
<th>Staining Intensity</th>
<th>Frequency/ Percentage</th>
<th>Mean ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>23 (51.1%)</td>
<td>8.2±1.43</td>
</tr>
<tr>
<td>Moderate</td>
<td>14</td>
<td>7.13±1.12</td>
</tr>
<tr>
<td>Severe</td>
<td>8</td>
<td>11.1±2.34</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>21 (46.7%)</td>
<td>13.14±5.27</td>
</tr>
<tr>
<td>Moderate</td>
<td>17</td>
<td>41.13±5.98</td>
</tr>
<tr>
<td>Severe</td>
<td>7</td>
<td>66.9±3.77</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The sixth most frequent cancer in the globe is HNSCC. OSCC continues to be a major health issue across the world. [16] Precancerous epithelium is the most common source of the disease, however it can also be caused by a preexisting malignant illness. The presence of metastases in regional lymph nodes, as well as a tumour with a deep invasive front, is all signs of a poor prognosis linked to the carcinoma itself. [17] For 30 years, OSCC morbidity and death rates have remained stagnant in spite of treatment advancements. [18] Oral cancers are the outcome of long-term accumulations of mutations that lead to the progression from normal tissue to cancerous tissue. For this reason, early detection and diagnosis of oral premalignant diseases (OPMD) may aid in the prevention of many OSCCs. An early diagnosis of high-risk OPMD might be a key to lowering the cost, mortality and morbidity of therapy related with SCC. Some OPMD exist prior to the creation of OSCC. Osf, leukoplakia, and erythroplakia tend to show up in the same places over and over.

Current retrospective study had 45 cases of OSF in which males were significantly greater than females. Most of the cases were between ages 18-40 years. Presented results of our study showed resemblance to the studies conducted in past.[19,20] Tobacco, smoking and areca nut were the most common causes of OSF in our research. A growing body of research suggests that betel nut chewing is a substantial contributor to OSF risk [21]. It's unfortunate that the professionally engineered areca nut is inexpensive, delicious, and easily available. A child's increased risk for addiction is exacerbated by parents' and children's lack of oral health literacy, increasing OSF's burden [22]. In the betel nut, arecoline is the primary component that begins the OSF process. Cell proliferation is accelerated by low concentrations of arecoline, but DNA damage, ROS, and LOX activity are increased at high concentrations of arecoline[23]. Inhibition of ataxia-telangiectasia-associated DNA repair by arecoline is another benefit of this compound. Arecoline treatment of cells increases LOX expression in oral cancer cells [24]. Chewing betel nuts has been connected to an increased risk of OSF, as has smoking, consuming alcohol, and chewing tobacco.[25,26] Tobacco chewed with areca nut was shown to be common among men in a research of 1000 cases.[27]

In our literature burning sensation and pain were the most prevalent symptoms among enrolled cases. It has been also studied in previous researches that burning, ulceration and pain were the most common symptoms among OSF patients a research conducted by Holla v et al in 2016.[28] OSF's growth and malignant transformation may be aided by hypoxia, and we hypothesised that HIF-1 may play a role. It was possible to count stained blood vessels and fibroblasts in several OSF samples using IHC and the hot-spot technique, which revealed a high overexpression of HIF-1. There was formerly thought to be a link between the number of blood vessels in OSF patients and HIF-1 expression, but this has subsequently been debunked. According to the findings of this investigation, HIF-1 expression was increased in patients with OSF due to an increase in fibroblast numbers that were greater than the number of blood vessels. High HIF-1 levels have been related to a number of chemicals that are thought to have a
role in carcinogenesis. VEGF is a key player in angiogenesis, a critical stage in carcinogenesis, and it must be taken into account as such. [29,30] According to Dunkel et al.[31], the CD44 low HIF-1 high signature was associated with shorter disease-free survival. Researchers Uehara et al.[32] revealed that the expression of HIF-1 in OSCC was significantly enhanced in Areca quid chewing-associated OSCC, according to Lee et al.[33]. Using reverse transcription-polymerase chain reaction, Zheng et al.[34] found an association between an increase in HIF-1 mRNA levels in tongue carcinomas and a higher pathological differentiation grade. HIF-1 protein expression was observed to be associated with lower disease recurrence and improved disease-free survival in Dos Santos et al. [35]'s study. An early event in prostate carcinogenesis was discovered to be HIF-1 overexpression, according to a recent study. [36] Compared to the normal prostatic epithelium, there was an increase in HIF-1 expression in cases of benign prostatic hyperplasia, prostatic intraepithelial hyperplasia, and malignancy.

In our study mean blood vessels were 11.1±2.34 with majority of the cases had mild staining intensity in 23 (51.1%) cases. Mean fibroblasts were 42.23±5.64 and had mild staining intensity in majority cases 21 (46.7%). Results of our study were comparable to the study conducted previously in Pakistan by Kanwal et al. in 2021.[37] It has become the focus of various investigations to look into the existence of molecular markers that may be used to diagnose OSF and the development of oral cancer early on and cure it. [38] Research suggests that HIF-1α may play a major role in fibrosis-induced carcinogenesis, and that it may even be the cause of fibrosis. Measuring the number of copies of a gene expressed per cell, tissue, or organism is a common practice in many branches of biology. RT-PCR, a highly sensitive and semiquantitative technology that is straightforward to conduct in a clinical laboratory, convenient and non-radioactive, and suited for regular testing, has several advantages. By using RT-PCR, mRNA precursors may be detected and quantitative gene expression levels can be calculated. [39]

CONCLUSION
We found that HIF-1α was an effective factor in the malignant transformation of oral submucous fibrosis. Aside from that, our findings revealed that OSF malignancy might be detected by the use of HIF-1 as a biomarker.

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