

Study of Hypoxia-inducible factor-2 α expression in the malignant transformation of Oral submucous fibrosis

Immanuel Joseph, Joshua Elizabeth, Umadevi K Rao, Kannan Ranganathan

Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, Tamil Nadu, India

Abstract

Context: Hypoxia-inducible factor (HIF)-2 α is overexpressed in primary and metastatic human cancers, whose expression is correlated with tumor angiogenesis and patient mortality. HIF plays a role in the progression of fibrosis in oral submucous fibrosis (OSF).

Aim and Objective: The aim is to study and compare the expression of HIF-2 α in OSF (a), oral squamous cell carcinoma (OSCC) with areca nut usage (b), OSCC without areca nut usage (c) and normal mucosa (d) by immunohistochemistry.

Subjects and Methods: Immunohistochemical detection of HIF-2 α was done on 51 samples, which included 11 cases (a), 15 cases (b), 15 cases (c) and the expression was compared with that of (d).

Statistical Analysis Used: Data were analyzed using the SPSS™ software (ver. 21.0). Chi-square test and kappa analysis were performed to compare the intensity of staining between the groups and for inter-observer agreement, respectively. Value of $P \leq 0.05$ was considered statistically significant. The mean labeling index between the groups was studied by the Kruskal–Wallis test.

Results: All the cases of (d), (a), (b) and (c) showed HIF-2 α expression ($P = 0.329$). About 13% cases of (c) showed intense expression ($P = 0.406$) and 27% of (a) cases showed expression only in the connective tissue ($P = 0.023$). The number of positively stained nuclei in both (b and c) cases reduced as the tumor progression was from well to poorly differentiated.

Conclusion: Areca nut initiates fibrosis and subsequent hypoxia in OSF which triggers HIF-2 α expression in the epithelium. HIF-2 α could be a surrogate marker for cancer initiation and progression.

Keywords: Hypoxia-inducible factor, malignant transformation, oral cancer, oral submucous fibrosis

Address for correspondence: Dr. Immanuel Joseph, 2/102, East Coast Road, Uthandi, Chennai - 600 119, Tamil Nadu, India.

E-mail: drjoseph 8287@gmail.com

Submitted: 02-Feb-2019, **Revised:** 04-Sep-2019, **Accepted:** 06-Dec-2019, **Published:** 08-May-2020

INTRODUCTION

Oral cancer is emerging as a major global problem. Head and neck cancer is the 11th most common cancer globally.^[1] Oral squamous cell carcinoma (OSCC) accounts for 90%–95% of all oral cancers.^[2] In India, one-fifth of all cancers reported are oral cancers, mainly attributed to tobacco consumption.^[1]

Areca nut (AN)/betel quid chewing causes oral submucous fibrosis (OSF), a potentially malignant disorder, with an overall prevalence rate of 6.42% in India and male-to-female ratio of 6.8:1.^[3] OSF shows extensive fibrosis, reduced vascularity, hypoxia and epithelial atrophy. Hypoxia is regulated by hypoxia-inducible factors (HIFs), helix transcription factors that mediate the adaptive

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DOI:

10.4103/jomfp.JOMFP_42_19

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How to cite this article: Joseph I, Elizabeth J, Rao UK, Ranganathan K. Study of Hypoxia-inducible factor-2 α expression in the malignant transformation of Oral submucous fibrosis. J Oral Maxillofac Pathol 2020;24:33-9.

responses to hypoxia. They are involved in carcinogenesis and tumor growth through the regulation of genes involved in angiogenesis, glycolytic metabolism and other biological mechanisms. HIFs have been reported to be involved in the malignant transformation of epithelia in the breast and prostate and in OSF.^[4] HIF-1 α , a HIF protein is over expressed in OSF which enhances the expression of a variety of other factors such as vascular endothelial growth factor (VEGF).^[5] HIF-2 α (also called EPAS1/HRF/HLF/MOP2) is a mammalian basic helix-loop-helix per-aryl hydrocarbon receptor nuclear translocator (ARNT) - Sim (bHLH-PAS) protein similar to HIF-1 α . Both HIF-1 α and HIF-2 α are closely related structurally, sharing 48% of overall amino acid identity.^[6]

HIF-2 α is expressed prominently in vascular endothelial cells during embryonic development, liver hepatocytes, kidney fibroblasts, epithelial cells of intestinal lumen, pancreatic interstitial cells, interstitial cells of heart myocytes and lung Type II pneumocytes. Further, it has been shown to be expressed in vascular cells, parenchymal cells and infiltrating macrophages in the tumor microenvironment. HIF-2 α binds to ARNT and its function is to transactivate the hypoxia-responsive genes namely, those which target erythropoietin and VEGF^[6] and may have an important role in tumorigenesis.^[7]

The present study was done to study the expression of HIF-2 α by immunohistochemistry in patients with OSF, OSCC in patients with a history of AN usage, OSCC in patients without a history of AN usage and normal oral mucosa.

SUBJECTS AND METHODS

Patients and tissue samples

Fifty-one archived formalin fixed paraffin embedded blocks from the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital were retrieved. The samples were Group 1 OSF ($n = 11$), Group 2 OSCC associated with AN habit (OSCC-AN) ($n = 15$), Group 3 OSCC without AN habit (OSCC-WAN) ($n = 15$), and Group 4 normal mucosa ($n = 10$). The study was approved by the Institutional Review Board of Ragas Dental College and Hospital.

Demographic details of all the patients were recorded which included age, gender, and history of any deleterious habit such as alcohol consumption, tobacco (chewing/smoking), and other products like AN. Normal controls were patients with no history of habits and who had an apparently normal mucosa clinically.

Immunohistochemical determination

Formalin fixed paraffin embedded serial tissue sections were cut to 5- μ m thickness and mounted on Superfrost APC coated slides. Antigen retrieval was achieved by transferring the slides to TRIS EDTA buffer of pH 9 and steamed in the pressure cooker at 15 lbs pressure for 15 min. Primary antibody namely, mouse monoclonal HIF-2 α antibody (clone ep-190b, abcam) diluted to 1:100 in tris-buffered saline was applied to sections and incubated for 90 min in a humid chamber. The sections were equilibrated to room temperature, washed with tris-buffered saline three times and then incubated for 30 min at room temperature with Poly Excel-HRP Micro polymer IHC detection system which was used as secondary antibody. Color was developed using DAB chromogen for 5 min. Sections were counter-stained with Harris hematoxylin, mounted and examined with a light microscope. Negative control sections were processed by omitting the primary antibody. Preeclamptic placental tissue known to be immunoreactive for HIF-2 α was used as positive control. The HIF-2 α immunoreactivity is mainly located in the nuclei of the syncytiotrophoblast, trophoblastic villous cells, and fetoplacental vascular endothelium in the preeclamptic villous placenta which is not regulated by hypoxia in placental villous explants [Figure 1].^[8,9] In breast epithelial tissue, under normoxic conditions, HIF-2 α expression is restricted to the cytoplasm, whereas in hypoxic conditions, the expression is seen both in the cytoplasm and in the nucleus.^[10]

Evaluation of slides

The staining intensity of the cytoplasm was analyzed in the basal, suprabasal layers of epithelium and connective tissue

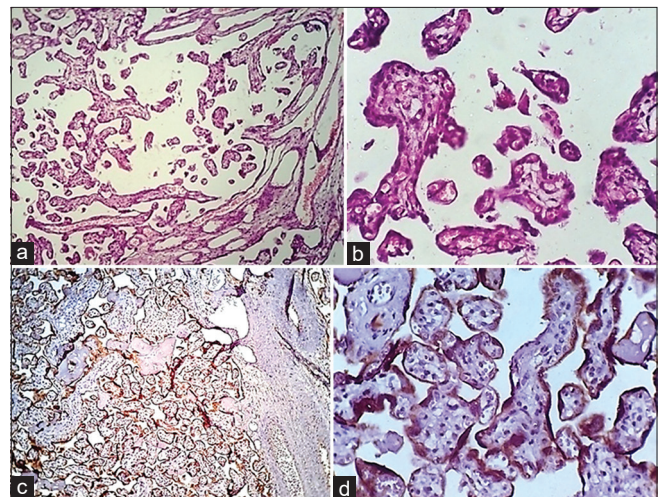


Figure 1: Histopathological image shows placental chorionic villi and blood vessels (H & E), ($\times 100$) (a) and ($\times 400$) (b) respectively, hypoxia-inducible factor-2 α staining the trophoblastic layer of the chorionic villi ($\times 100$) (c) and ($\times 400$) (d) respectively

in the study groups. Each case was graded as (–) nil or the absence of stain, (+) mild, (++) moderate, and (+++) intense stain by two-blinded observers independently with respect to the positive control.^[11]

Nuclei of cells expressing HIF-2 α were counted (1000 cells/entire section) in the basal and suprabasal layers of controls, OSF, OSCC-AN, and OSCC-WAN. Percentage of such positive cells was categorized as (0) no expression; (1) $\leq 20\%$ of cells positive; (2) 20%–50%; (3) $\geq 50\%$.

The mean labeling index (MLI) for all the positive groups was calculated using the formula:

$$\frac{\text{Number of positive cells}}{\text{Total number of cells}} \times 100$$

Statistical analysis used

Data were entered and analyzed using SPSS™ Inc. (Ver. 21.0, IBM, Chicago, Illinois, US). Pearson's Chi-square test was done to compare the intensity of staining between the groups. Value of $P \leq 0.05$ was considered statistically significant. Kappa analysis was performed to compare the intensity of HIF-2 α staining interobserver agreement between two observers ($\kappa = 0.92$). The MLI between the groups was studied by the Kruskal–Wallis test.

RESULTS

Subjects

The study participants were predominantly males ($P = 0.05$). Majority of patients were in 40–60 years age group. About

36% of OSF and 40% of OSCC-AN cases were in the age group of 20–40 years and 7% of OSCC-WAN cases were in the age group of 20–40 years ($P = 0.006$) described in Table 1. Among the study groups, in Group 2 (OSF), four samples got washed off during the immunohistochemical process.

Hypoxia-inducible factor-2 α staining

Expression of HIF-2 α was seen in all the four groups ($P = 0.329$) [Table 1]. The pattern of staining was either cytoplasmic alone or cytoplasmic and nuclear in all the study groups [Table 2] ($P = 0.23$). Only in 27% of OSF cases [Table 3], the expression was seen only in the connective tissue and not in the epithelium ($P = 0.023$).

Table 4 describes the overall intensity of staining as well as staining with respect to the localization of tissue. Mild expression was seen in 45% of OSF cases, 54% of OSCC-AN, 47% of OSCC-WAN, and 60% of control group cases. Moderate expression was seen in 55% of OSF cases, 33% of OSCC-AN, 40% of OSCC-WAN and 30% of the control group. Intense expression was seen in 13% of OSCC-AN cases ($P = 0.406$).

The intensity of staining in the basal layer showed moderate expression in 13% of OSCC-AN and 6% of OSCC-WAN cases and intense expression only in 7% cases of OSCC-AN ($P = 0.682$). The intensity of staining in the suprabasal layer showed mild expression in 27% of OSF cases, 47% of OSCC-AN and OSCC-WAN cases and 70% of controls. Forty-six percent of OSF cases, 33% of

Table 1: Baseline characteristics of study groups

Demographic details	Group 1 (n=11) (%)	Group 2 (n=15) (%)	Group 3 (n=15) (%)	Group 4 (n=10) (%)	P
Male	82	100	67	60	0.05*
Female	18	0	33	40	
20–40 years	36	40	7	90	0.006*
41–60 years	55	47	66	10	
61 years and above	9	13	27	0	
Habits					
Areca nut	82	100	0	0	0.000*
Tobacco only	0	0	13	0	
Tobacco+alcohol	18	0	0	0	
No habits	0	0	87	100	
Overall staining – HIF-2 α					
Present	100	100	87	90	0.329
Absent	0	0	13	10	

* $P \leq 0.05$ is significant. HIF: Hypoxia-inducible factor

Table 2: Distribution of staining pattern of hypoxia-inducible factor-2 α among the study groups

Staining pattern of expression	Group 1 (n=11) (%)	Group 2 (n=15) (%)	Group 3 (n=15) (%)	Group 4 (n=10) (%)	P
Cytoplasmic	55	53	67	40	0.234
Cytoplasmic+nuclear	18	47	20	50	
No expression	27	0	13	10	

Table 3: Hypoxia-inducible factor-2 α expression with respect to tissue localisation in the study groups

Tissue localisation	Group 1 (n=11) (%)	Group 2 (n=15) (%)	Group 3 (n=15) (%)	Group 4 (n=10) (%)	P
Overall expression	100	100	87	90	0.023*
Epithelium					
Suprabasal alone	18	33	7	10	
Basal+suprabasal alone	0	20	20	50	
Connective tissue					
Connective tissue alone	27	0	0	0	
Combined expression (epithelium+connective tissue)					
Suprabasal+connective tissue	0	0	7	10	
Basal+suprabasal+connective tissue	55	47	53	20	

* $P \leq 0.05$ is significant**Table 4: Tissue localisation and intensity of expression among the study groups**

Intensity	Group 1 (n=11) (%)	Group 2 (n=15) (%)	Group 3 (n=15) (%)	Group 4 (n=10) (%)	P
Overall intensity					
-	0	0	13	10	0.406
+	45	54	47	60	
++	55	33	40	30	
+++	0	13	0	0	
Basal layer					
-	45	33	27	30	0.682
+	55	47	67	70	
++	0	13	6	0	
+++	0	7	0	0	
Supra basal layer					
-	27	0	13	10	0.268
+	27	47	47	70	
++	46	33	33	10	
+++	0	20	7	10	
Connective tissue					
-	18	53	40	70	0.319
+	46	13	40	20	
++	36	27	13	10	
+++	0	7	7	0	

-: No expression, +: Mild expression, ++: Moderate expression, +++: Intense expression

OSCC-AN and OSCC-WAN cases and 10% of controls showed moderate expression. Intense expression was seen only in 20% of OSCC-AN, 7% of OSCC-WAN and 10% of control group ($P = 0.268$). Intensity of staining in the connective tissue showed mild expression in 46% of OSF cases, 13% of OSCC-AN, 40% of OSCC-WAN and 20% of control group. Moderate expression was seen in 36% of OSF, 27% of OSCC-AN, 13% of OSCC-WAN and 10% of controls. Intense expression was seen only in (7%) OSCC-AN and (7%) OSCC-WAN cases, whereas none of the cases of OSF and control group showed intense expression in the connective tissue ($P = 0.319$).

Hypoxia-inducible factor-2 α positive stained cells and grades of differentiation (oral squamous cell carcinoma)

Well-differentiated OSCC-AN group showed 20%–50% of positive stained cells in 44% of cases and >50% of positive stained cells in 11% of cases, whereas moderately differentiated SCC showed both <20% of positive stained cells and 20%–50% positive stained cells in 17% of cases

($P = 0.34$) [Table 5]. Well-differentiated OSCC-WAN group had 10% of cases showing 20%–50% of positive stained cells and 20% of cases showing >50% of positively stained cells.

Mean labeling index among study groups

The MLI of HIF-2 α staining between the groups [Table 6]; OSF, OSCC-AN, OSCC-WAN and controls were 10.975 ± 19.21 , 18.49 ± 22.88 , 7.85 ± 18.08 and 12.24 ± 24.08 , respectively. The difference between the four groups was noted as $P = 0.282$.

DISCUSSION

OSF is a chronic fibrotic potentially malignant disorder particularly prevalent in the Indian subcontinent.^[12] OSF is histopathologically characterized as fibrosis of the subepithelial connective tissue. This chronic fibrotic condition is brought about by the accumulation of collagen Type 1 and impaired degradation of collagen, reduced vascularity in the connective tissue and hypoxia.^[5]

Table 5: Distribution of HIF-2 α positive stained cells in grades of OSCC-AN and OSCC-WAN

OSCC-AN (Group 2)				
Percentage of positive stained cells	Well differentiated OSCC (n=9) (%)	Moderately differentiated OSCC (n=6) (%)	Poorly differentiated OSCC (n=0) (%)	P
0	44	67	0	0.343
1	0	17	0	
2	44	17	0	
3	11	0	0	
OSCC-WAN (Group 3)				
Percentage of positive stained cells	Well differentiated OSCC (n=10) (%)	Moderately differentiated OSCC (n=2) (%)	Poorly differentiated OSCC (n=3) (%)	P
0	70	100	100	0.759
1	0	0	0	
2	10	0	0	
3	20	0	0	

0: No expression, 1: <20% positive stained cells, 2: 20%–40% positive stained cells, 3: >40% positive stained cells. OSCC: Oral squamous cell carcinoma, OSCC-AN: OSCC with areca nut, OSCC-WAN: OSCC without areca nut

Table 6: Comparison of hypoxia-inducible factor-2 alpha mean labeling index between the study groups

Groups	n	Mean \pm SD	Overall P
Group 1 versus 2	2	10.975 \pm 19.21	0.282
	7	18.49 \pm 22.88	
Group 1 versus 3	2	10.975 \pm 19.21	
	3	7.85 \pm 18.08	
Group 1 versus 4	2	10.975 \pm 19.21	
	4	12.24 \pm 24.08	
Group 2 versus 3	7	18.49 \pm 22.88	
	3	7.85 \pm 18.08	
Group 2 versus 4	7	18.49 \pm 22.88	
	4	12.24 \pm 24.08	
Group 3 versus 4	3	7.85 \pm 18.08	
	4	12.24 \pm 24.08	

SD: Standard deviation

The clinicopathological profile of OSCCs arising in a preexisting OSF is different from those arising due to other etiological factors. One of the key differences reported is the rate of lymph node metastasis which is significantly lower in OSCCs arising from preexisting OSF.^[13] The hypoxia-responsive genes (HIF-1 α and HIF-2 α) regulate the cellular response to hypoxia and are important for solid tumor growth, survival and promotion of aggressiveness. Overexpression of HIF-2 α is associated with poor prognosis in lung, colon and ovarian cancers.^[14]

In our study, we observed that the majority of OSF and OSCC-AN cases were in the age group of 20–40 years. Our finding was similar to the study by Chaturvedi *et al.* and Balaji *et al.*, where most patients had OSCC with OSF belonging to the younger age group.^[15,16]

Studying the pattern of HIF-2 α staining [Table 2], the highest number of cases among the study groups, which showed nuclear and cytoplasmic staining, was seen in the OSCC-AN group (100%). There was no significant difference in the pattern of staining between the groups. Pahlman *et al.* reported that HIF-2 α expression was seen

both in the nucleus and in the cytoplasm in hypoxic regions, but cytoplasmic staining was not always accompanied by nuclear staining. This is because in normoxic condition, HIF-2 α is regulated by prolyl hydroxylase domain enzymes (PHDs) that initiate its degradation through the von Hippel–Lindau protein (tumor suppressor protein). In hypoxic conditions, the activity of PHDs is inhibited and HIF-2 α is not degraded but enters the nucleus and binds to a conserved DNA sequence (hypoxia-responsive element) and initiates the transactivation of hypoxia-responsive factors (nuclear factor- κ B, AP-1 [activator protein 1], p53 and c-Myc), apart from the HIF family. There is a crosstalk mechanism between these factors, determined by the duration and intensity of hypoxia exposure, which results in a coordinated cellular response. The promoted activity of c-Myc in cell lines is found due to an elevated HIF-2 α .^[11,17]

All the cases from OSF and OSCC-AN groups showed the expression of HIF-2 α [Table 3]. Twenty percent of ($n = 15$) OSCC-AN cases showed expression in the basal and suprabasal layers of the epithelium, whereas in 33% of ($n = 15$) cases, expression was present in the suprabasal layer alone. This reflects the findings of Pahlman *et al.* in mammary epithelial cells where they found the highest HIF-2 α expression in the most differentiated cells, i.e., the lactating cells than in virgin mouse breast epithelium.^[10] In the connective tissue, there was a significant difference in HIF-2 α expression, since it was seen only in OSF cases. Fraisl *et al.* have shown that hypoxic stress activates HIF-2 α in endothelial cells and it plays a significant role in vascular morphogenesis, its integrity and the assembly by restoring the oxygen supply required for cellular metabolism.^[18] The extensive fibrosis and hyalinization in OSF could be alterations in the vasculature brought about by HIF-2 α in the connective tissue stroma of OSF.

Comparing the intensity of HIF-2 α among the study groups [Table 4], all the cases from OSF and OSCC-AN had expressed HIF-2 α and most cases showed mild to moderate intensity. There was intense expression seen only in OSCC-AN cases [Figure 2]. This intense HIF-2 α expression in AN users was similar to that of Koukourakis *et al.*, who stated that carbonic anhydrase 9 (CA9), a hypoxia-inducible transmembrane enzyme has been shown to correlate with direct measurement of oxygen tension in cervical cancer. Two factors regulate the different pathways of hypoxia, namely CAIX and HIF-2 α . Hypoxia-inducible CAIX is regulated by HIF-1 and HIF-2 α . There was a significant association of high reactivity of HIF-2 α and CAIX seen with poor loco-regional control in head and neck SCCs. Based on our finding of intense expression in OSCC-AN cases, AN could have assisted in the pathogenesis of OSCC in association with CAIX which is also found in AN chewers.^[19]

In the basal layer of epithelium, most cases of OSF and the control group showed only mild expression [Table 4]. The cases of OSCC-AN and OSCC-WAN showed mild and moderate intensity, whereas intense expression was seen only in the OSCC-AN group. When the suprabasal layer was observed for the intensity of expression, most cases of the study groups including the controls showed varied expression – mild (27% OSF, 47% OSCC-AN and OSCC-WAN, 70% controls), moderate (46% OSF, 33% OSCC-AN and OSCC-WAN, 10% controls) and intense (20% OSCC-AN, 7% OSCC-WAN, 10% controls) expression. This observation is similar to the study by Pahlman *et al.*, who suggests that HIF-2 α can accumulate and get activated in response to hypoxic stress. It has been shown in breast epithelium that HIF-2 α is associated with breast cancer metastasis and poor patient survival and has also been reported that hypoxia and the transcriptional activity are linked to a state of loss of polarization and a cancer-like phenotype in primary human breast epithelial

cells.^[11] Based on these findings, we extended the analogy to the oral epithelium and postulated that the intense expression of HIF-2 α though not statistically significant, in OSF and OSCC-AN cases, can be a marker of malignant transformation.

The number of positively stained nuclei in OSCC-AN and OSCC-WAN cases in relation to tumor differentiation is shown in Table 5. As the tumor progressed from well to poor differentiation, there was a reduction in the number of positive stained nuclei and also there was no correlation with the intensity of HIF-2 α . This was consistent with the findings of Talks *et al.*, where the intensity of expression in breast, pancreatic and prostatic adenocarcinomas did not correlate with the number of positive tumor nuclei which ranged from <1% to 95% of tumor cells.^[20]

The MLI [Table 6] of HIF-2 α in OSF cases was 10.975 ± 19.21 , mean LI of OSCC-WAN 7.85 ± 18.08 and the mean LI of OSCC-AN was 18.49 ± 22.88 . The mean LI of OSCC-AN cases was not significantly higher than the other groups.

In our study, we had significant differences in HIF-2 α expression, in both OSF and OSCC-AN groups. The former group had patients expressing HIF-2 α in the connective tissue alone, and the latter group had patients showing intense expression. We did not arrive at significant differences between the groups for all the other parameters evaluated. This could be due to the small number of cases in this study. Hence, the expression of HIF-2 α , need to be studied with a larger sample size to confirm its role in malignant transformation of OSF.

CONCLUSION

In our study, we have highlighted the differences of HIF-2 α in the study groups. Altered HIF-2 α expression,

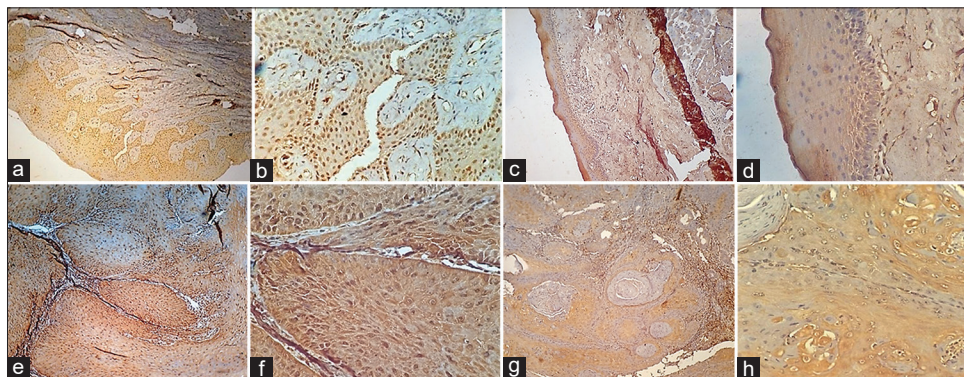


Figure 2: Histopathological image shows expression of hypoxia-inducible factor-2 α in normal mucosa ($\times 100$) (a), ($\times 400$) (b); in oral submucous fibrosis ($\times 100$) (c), ($\times 400$) (d); oral squamous cell carcinoma with areca nut ($\times 100$) (e), ($\times 400$) (f); oral squamous cell carcinoma without areca nut ($\times 100$) (g), ($\times 400$) (h)

due to the effect of AN on tissues could be an indication for malignant transformation and influence its prognosis.

Acknowledgement

We acknowledge the Tamil Nadu Dr. MGR Medical University, Chennai for their constant encouragement toward Research work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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