Immunohistochemical Expression of HIF-1α in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma

Gunveen Kour[1], Swati Parhar[2], Diviani Feria[3], Tanu[4], Divya[5], Vivek Singh Dahiya[6]

[1]Senior Resident, Institute of Dental Sciences Sehora Jammu, J & K, India;
[2]Associate Professor, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Barwala, Panchkula, Haryana, India
[3,5]PG student , Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Barwala, Panchkula, Haryana, India
[4]Senior Lecturer, , Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Barwala, Panchkula, Haryana, India
[6]Professor & HOD, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Barwala, Panchkula, Haryana, India

Corresponding author: Dr. Swati Pahar**,** Associate Professor, Department of Oral Pathology, Swami Devi Dyal Hospital and Dental College, Barwala, Panchkula, Haryana, India

ABSTRACT

Background: Hypoxia is one of the hallmarks of cancer and has been demonstrated in different types of solid tumors including head and neck squamous cell carcinoma. The present study was immunohistochemical evaluation of Hif 1 alpha in OSMF and OSCC.

Materials & Methods: Histopathologically diagnosed 20 cases of oral submucous fibrosis and oral squamous cell carcinoma each. The immunohistochemistry was carried out on neutral buffered formalin-fixed paraffin-embedded tissue sections by using the monoclonal antibody of HIF-1α.

Results: In group 1, there are six slides (10%) out of 20 showing intense reaction with HIF-1 alpha (score 3), ten slides (30%) out of 20 showing moderate reaction with HIF-1 alpha (score 2), two slides (10%) out of 20 showing mild reaction with HIF-1 alpha (score 1) and two slides (10%) out of 20 showing no colour reaction with HIF-1 alpha (score 0). In group 2 (Squamous cell carcinoma) there are fifteen slides (75%) out of 20 showing intense reaction with HIF-1 alpha (score 3) and five slides (25%) out of 20 showing moderate reaction with HIF-1 alpha (score 2).

Conclusion: Rise in expression of HIF-1a from OSMF to OSCC. Upregulation of HIF-1a is positively correlated with oral carcinogenesis. Thus, it can be used as both diagnostic as well as a prognostic marker in the patients with OSMF and OSCC.

Key words: Hematoxylin, Eosin, Oral submucous fibrosis

Tob Regul Sci.™ 2021;7(6-1): 7623-7633
DOI: doi.org/10.18001/TRS.7.6.1.81

Introduction

Hypoxia is one of the hallmarks of cancer and has been demonstrated in different types of solid tumors including head and neck squamous cell carcinoma.[1] It reflects the imbalance between oxygen consumption by the rapidly proliferating cancer cells and the insufficient oxygen delivery due to poor vascularization and blood supply.[2]

Oral Submucous fibrosis is a chronic, insidious and progressive oral mucosal disease that primarily affects any part of the oral cavity.[3] It is characterized by a juxta-epithelial inflammatory reaction followed by progressive fibrosis of lamina propria and the underlying sub mucosal layer, with associated epithelial atrophy. This leads to restricted mouth opening, resulting trismus.[4] In addition, the over expression of hypoxia-induced factor-1α is seen in OSMF, which indicates changes in cell proliferation, maturation and metabolic adaptation, increasing the possibility of malignant transformation. It carries a high risk of transition to oral squamous cell carcinoma.[5]

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy accounting for 94% of all malignant neoplasms of the oral cavity. Over past two decades, research has been focused on the identification of a molecular marker that can be used to predict the role of hypoxia in oral submucous fibrosis and oral squamous cell carcinoma.[6] One such marker that has been used successfully is Hypoxia Inducible Factor-1alpha (HIF-1α). It is an oxygen sensitive master transcription factor that is stabilized in hypoxic conditions, after which it translocate into the nucleus to interact with hypoxia responsive elements. It is a key regulator responsible for the induction of the genes that facilitate adaptation and survival of cells from normoxia to hypoxia.[7]

Overexpression of HIF-1α has recently been linked with unfavourable prognosis in several types of malignant tumours. However, definitive evidence of this association is yet to be obtained. Hence this study was undertaken to evaluate the immunohistochemical expression of HIF-1α in histological sections of oral submucous fibrosis and oral squamous cell carcinoma. The present study was immunohistochemical evaluation of Hif 1 alpha in OSMF.

Materials & Methods

This study was carried out in the department of Oral Pathology and Microbiology of Swami Devi Dyal Dental Hospital and College, Barwala. Inclusion criteria for oral submucous fibrosis group was blocks of histologically proved oral submucous fibrosis and patient age between 21 to 65 years. Exclusion criteria were cases showing evidence of malignancy / micro invasion, cases which cannot be diagnosed histologically, cases where epithelium is not seen histologically and slides with tissues which are not representative of the pathology. Inclusion criteria for oral squamous cell carcinoma group, blocks of histologically proved oral squamous cell carcinoma and patient age between 21 to 75 years. Exclusion criteria were cases showing extensive necrosis histologically and cases which cannot be diagnosed histologically.

The study specimens included were categorized as follows, group 1containing 20 specimens oral submucous fibrosis, group 2 containing 20 specimens of oral squamous cell carcinoma and 10 normal oral mucosal biopsies were used to investigate the expression of HIF-1a by immunohistochemistry . Data on patient age, gender and lesion site were obtained from the biopsy requisition forms submitted. The tissue sections thus obtained were stained using the following methods such as standard Hematoxylin and Eosin (H&E) Stain and immunohistochemical staining using primary antibody (HIF-1α) stain. Data thus obtained were subjected to statistical analysis. P value < 0.05 was considered significant.

IMMUNOSTAINING EVALUATION

A. Quality Score:

- Score 0 (no colour reaction)
- Score 1 (mild reaction)
- Score 2 (moderate reaction)

- Score 3 (intense reaction)

B. Quantity Score:

- Score 0: (no positive cells)
- Score 1: (<10% of positive cells)
- Score 2: (10-50% positive cells)
- Score 3: (51-80% positive cells)
- Score 4: (>80% positive cells)

Then the Scores for the expression intensities (quality score)i.e A and percentage of positive cells (quantity score)i.e B were multiplied to calculate the immunoreactive score. (A×B= IRS)

- Negative: (0-1)
- Mild: (2-3)
- Moderate: (4-8)
- Strongly positive: (9-12)

Statistical analysis:

The Normality of the variables (Quantitative data) was tested with the Shapiro-Wilk test / Kolmogorov Smirnov tests of Normality. As our data for scoreswas skewed so scores were written in the form of its Mean, SD, median and interquartile range, Group comparisons of values of data were made with Mann-Whitney test for 2 groups, Age was normally distributed so it was compared with t-test and was presented as mean, SD with range. Gender was reported as counts and percentages. Group comparisons were made with the Chi-Sqtest.A P value < 0.05 was considered significant. All the statistical tests were two-sided and were performed at a significance level of α=.05. Analysis was conducted using IBM SPSS STATISTICS (version 22.0).

Results:

On comparing HIF-1 alpha expression in OSMF and OSCC a gradual increase was found in its intensity score from OSMF to OSCC. Staining intensity scores of Group 1 (Oral submucous fibrosis) and Group 2 (squamous cell carcinoma) the maximum cases of group 1 exhibited moderate reaction with HIF-1 alpha (score 2)whereas majority of cases of group 2 exhibited intense reaction with HIF-1 alpha (score 3). (Table 1)

Table 1 Comparison of staining intensity score between group 1 and group 2

HIF-1 ALPHA* GROUP CROSSTABULATION				
STAINING INTENSITY SCORE		**STUDY GROUPS**		
		GROUP 1	**GROUP 2**	
Score 0 (no colour reaction)	Count	02	0	02
	% within group	10%	0.0%	10%
Score 1 (mild reaction)	Count	02	0	02
	% within group	10%	0.0%	10%
	Count	10	5	15

Score 2 (moderate reaction)	% within group	50%	25%	37%
Score 3 (intense reaction)	Count	06	15	21
	% within group	30%	75%	52%
TOTAL	Count	20	20	40
	% within group	100.0%	100.0%	100.0%

On comparing HIF-1 alpha expression in OSMF and OSCC a gradual increase was found in its quantity score from OSMF to OSCC. Staining quantity score of Group 1(Oral submucous fibrosis)and Group 2(squamous cell carcinoma) the maximum cases of Group 1 (Oral submucous fibrosis) exhibited 50-80% positively cells (score 3) whereas majority of cases of Group 2 (well differentiated squamous cell carcinoma) exhibited > 80% of positive cells (score 4) towards HIF-1 alpha. (Table 2). No expression of HIF-1 a was seen in normal mucosal biopsies samples (fig 5)

Table 2: Comparison of staining quantity score between group 1 and group 2

GROUP CROSSTABULATION				
STAINING QUANTITY SCORE		STUDY GROUPS GROUP 1	GROUP2	TOTAL
Score 0 (no positive cells)	Count	2	0	2
	% within group	10.0%	0.0%	5.0%
Score 1 (<10% of positive cells)	Count	5	0	5
	% within group	25.0%	0.0%	12.5%
Score 2 (10-50% positive cells)	Count	10	9	19
	% within group	50.0%	45.0%	47.5%
Score 3 (51-80% positive cells)	Count	3	11	14
	% within group	15.0%	55.0%	35.0%
Score 4 (>80% positive cells)	Count	20	20	40
	% within group	100.0%	100.0%	100.0%

Immunoreactive score (IRS) of Group 1 (Oral submucous fibrosis) and Group 2 (squamous cell carcinoma)the maximum cases of Group 1 (Oral submucous fibrosis) exhibited moderate expression (score 6) whereas majority of cases of Group 2 (well differentiated squamous cell carcinoma) exhibited strongly positive expression (score 12) towards HIF-1 alpha.(Table3)

Table 3: Comparison of IRS score between group 1 and group 2

GROUP CROSSTABULATION				
IRS SCORE		STUDY GROUPS		TOTAL
		GROUP 1	GROUP 2	
0	Count	2	0	2
	% within Group	10.0%	0.0%	5.0%
2	Count	2	0	2
	% within Group	10.0%	0.0%	5.0%
4	Count	3	0	3
	% within Group	15.0%	0.0%	7.5%
6	Count	7	5	12
	% within Group	35.0%	25.0%	30.0%
9	Count	3	4	7
	% within Group	15.0%	20.0%	17.5%
12	Count	3	11	14
	% within Group	15.0%	55.0%	35.0%
Total	Count	20	20	40
	% within Group	100.0%	100.0%	100.0%

Statistical analysis shows that Correlation between immunoreactive scores of group 1 and group 2 is significant. (p value=.034) (Table 5)

Table 5 Results of chi-square test

Chi- square test	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.048[a]	5	.034*
Likelihood Ratio	15.042	5	.010
Linear-by-Linear Association	10.970	1	.001
No. of Valid Cases	40		

Discussion

Hypoxia causes cell death if it is severe or prolonged. However, cancer cells acclimatize to this hostile environment. Protection against hypoxia in solid tumors is an important step in tumor development and progression. With the progression of the disease process of OSMF, the production of collagen type 1 is increased and the degradation of collagen is reduced by up to 75%.[8] Extensive fibrosis of the connective tissue causes reduction of vascularity, resulting in subsequent hypoxia in both fibroblasts and surface epithelium that causes atrophy and ulceration of the epithelium by inducing apoptosis.

Physiologists and clinicians define hypoxia as a state of reduced O_2 availability or decreased O_2 partial pressures below critical thresholds, thus restricting or even abolishing the function of organs, tissues or cells. In solid tumors, oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels and disturbed microcirculation. Development of hypoxic microenvironment is caused by the imbalance between oxygen consumption and oxygen delivery. The rapidly proliferating head and neck squamous cell carcinoma has insufficient vascularization with poor blood supply. Limiting blood supply network in the rapidly proliferating tumor region limits oxygen diffusion, resulting in the development of the hypoxic region. These areas with very low oxygen partial pressures exist in solid tumors, occurring either acutely or chronically. These microregions are heterogeneously distributed within the tumor mass and may be located adjacent to regions with normal O_2 partial pressures. The hypoxic stress stimulates solid tumor to upregulate expression of a variety of oncogenes such as HIF and vascular endothelial growth factor, which enhance irregular vascular endothelial cell proliferation and differentiation.

Despite several diagnostic and therapeutic advances over the last decades, the rate of mortality and 5-year survival of patients with OSCC has not been improved markedly. The incidence and mortality of OSCC are higher in developing countries. It has the potential for rapid and unlimited growth. Furthermore, hypoxia is a common feature that contributes to local and systemic cancer progression, resistance to therapy and poor outcome.[9]

In our study a total of 40 sample size was taken, which was categorised in two Groups namely Group 1 which comprised of 20 cases of histologically diagnosed cases of Oral submucous fibrosis and Group 2 which comprised of 20 cases of histologically diagnosed cases of Oral squamous cell carcinoma and 10 cases of normal oral mucosal biopsies were taken as control.

Mean age of whole sample was 45±13.048. Further, in our study, mean age of patients of Oral submucous fibrosis is 42±12.782 years and Oral squamous cell carcinoma is 48±12.929 years. According to study conducted by Hande A H et al[10], mean age of patient of Oral submucous fibrosis is 32.58 years.

In our study, males predominance is seen with 27 (67.5%) males of total sample and ratio. Group 1 (Oral submucous fibrosis) comprised of 14 (70%) males and 6 (30%) females while Group 2 (squamous cell carcinoma) comprised of 13 (65%) males and 7(35%) females. In similarity to ours Ribeiro M et al[11], also reported male predominance (76.3%), with mean age 56.3 years.

In Group 1 (Oral submucous fibrosis) two out of 20 specimens (10%) showed negative staining for HIF-1α, 2 out of 20 specimens (10%) were weakly stained, 10 out of 20 specimens (50%) showed moderate HIF-1α expression, and in 6 specimens out of 20 specimens (30%) HIF-1α was strongly expressed. Thus, our results showed that moderate HIF-1α expression was the most predominant pattern in Oral submucous fibrosis in our study with 50% cases followed by strong expression in 30% cases. These findings are in accordance with the previous studies done by Chaudhary M et al.[13]

In Group 2 (squamous cell carcinoma) 5 out of 20 specimens (25%) showed moderate HIF-1α expression, and in 15 specimens out of 20 specimens (75%) HIF-1α was strongly expressed. Hence, predominantly a strong HIF-1 alpha expression was exhibited by the OSCC specimens in our study. On comparison, a statistically significant difference is observed on comparison of staining intensities of both the groups (p=.023). Similar results of HIF-1alpha staining intensity was observed by Chaudhary M et al.[13]

In the present study quantitative expression of HIF-1 alpha or the percentage of immunopositive tumor cells in Group 1 (Oral submucous fibrosis) shows that, 3 out of 20 slides (15%) have >80% positive

cells, 10 out of 20 slides (50%) have 51-80% positive cells, 5 out of 20 slides (25%) have 10-50% positive cells and two out of 20 slides (10%) have no positive cells. Thus, predominently 51-80% of immunopositive tumor cells were seen in Oral submucous fibrosis in our study with 50% cases followed by 10-50% of immunopositive tumor cells in 25% cases. In Group 2 (Well differentiated squamous cell carcinoma) 11 out of 20 slides (55%) have >80% positive cells, 9 out of 20 slides (45%) have 51-80% positive cells. Hence, predominantly >80% of immunopositive tumor cells were seen in OSCC specimens in our study.

On comparison, a statistically significant difference is observed on comparison of staining quantities of both the Groups. These findings were in accordance with the previous studies conducted by Ribeiro M et al.[11] According to the study conducted by Brennan[14], the nuclear HIF-1a labelling indices in OSCC samples was significantly correlated with clinical staging of OSCCs. High expression of HIF-1α is defined based on a combination of the percentage of stained cells and the intensity of staining. Thus, scores of the percentage of immunopositive cells and cellular expression intensity is multiplied to calculate an immunoreactive score (IRS).

In our study, in Group 1 (Oral submucous fibrosis) two slides out of 20 (10%) exhibit negative expression, two slides out of 20 (10%) exhibit mild expression, ten slides out of 20 (50%) exhibit moderate expression and 6 slides out of 20 (30%) exhibit strongly positive expression towards HIF-1alpha. Hence, overall predominantly moderate HIF-1alpha expression was seen in OSMF in our study with 50% of the cases. In Group 2 (Well differentiated squamous cell carcinoma) five slides out of 20 (25%) exhibit moderate expression and 15 slides out of 20 (75%) exhibit strongly positive expression towards HIF-1 alpha. Hence, overall predominantly strong HIF-1alpha expression was exhibited by OSCC specimens in our study.

On comparing the immunoreactive scores of both the groups: Group 1 and Group 2 (OSMF AND OSCC) a statistically significant difference is observed. (p value=.034). Since statistically significant over all staining intensity (p=.003), percentage of immunopositive cells (p=.001) and immunoreactive scoring (p=.001) was obtained by HIF-1 alpha in Group 1(Oral submucous fibrosis) and Group 2 (squamous cell carcinoma). Thus, hypoxia can be considered an independent adverse prognostic factor in patients with Oral submucous fibrosis and Oral squamous cell carcinoma.

From our study we came to an inference that hypoxia may play a vital role in tumorigenesis. In our study, HIF-1alpha is found to be upregulated in OSCC in comparison to OSMF. A sequential upregulation of HIF-1alpha expression is observed from OSMF to OSCC suggesting a possible role of HIF-1alpha in oral carcinogenesis. It may also act as a marker for early detection of malignant transformation with prognostic significance. It can be targeted as a therapeutic agent to prevent the malignant conversion of OSMF into OSCC and can be considered as new auxillary method that will cause fewer complications. However, further studies are recommended for more reliable results to be reached.

Conclusion

This study confirmed the greater expression HIF-1a in oral squamous cell carcinoma as compared to oral submucous fibrosis. A progressive increase in the HIF-1a was observed in OSMF to OSCC. Hypoxia appears to be a key factor in regulating this effect.

OSMF ➡ HYPOXIA ➡ ANGIOGENESIS ➡ OSCC

Upregulation of HIF-1a is positively correlated with oral carcinogenesis. Hence, HIF-1a can be used as both diagnostic as well as a prognostic marker in the patients with OSMF and OSCC. To conclude,

we feel this biomarker can be utilized as a major asset for early detection of cancer as its role in tumorigenesis is evident.

References

1. Mills C, Joshi S and Niles R. Expression and function of hypoxia inducible factor 1 alpha in human melanoma under non hypoxic conditions. Molecular Cancer 2009; 8:104.
2. Semenza GL. Targetting HIF-1 for cancer therapy. Nat Rav Cancer 2003 Oct; 3(10):721-32.
3. Masoud G N, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B 2015 Sep; 5(5): 378–389.
4. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. Proc Natl Acad Sci USA. 1991; 88:5680–5684.
5. Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science. 1988; 242:1412–1415.
6. Wang GL, Jiang B, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix loop-helix-PA Shetero dimer regulated by cellular O2 tension. Proc Natl Acad Sci USA. 1995;92:5510-14.
7. Yang J, Zhang L, Erbel PJ, Gardner KH, Ding K, Garcia JA, Bruick RK. Functions of the Per/ARNT/Simdomainsofthehypoxia-induciblefactor.JBiolChem.2005;280:3604754.9 .C
8. Huang L and Bunn H. Hypoxia-Inducible Factor and Its Biomedical Relevance. JBC Mini Review 2003 as Manuscript R200030200.
9. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Embo J, 20 5197-5206.
10. Hande AH, Chaudhary M, Gadbail A, Zade P, Gawande M, Patil S. Role of Hypoxia in Malignant Transformation of Oral Submucous Fibrosis. J Datta Meghe Inst Med Sci Univ 2018; 13: 38-43.
11. Ribeiro M, Teixeira S, Azevedo M, Jr A, Gontijo A and Vêncio E. Expression of hypoxia-induced factor-1 alpha in early-stage and in metastatic oral squamous cell carcinoma. Tumor Biology April 2017: 1–8
12. Tilakaratne W M et al. Upregulation of HIF-1a in malignant transformation of oral submucous fibrosis. J Oral Pathol Med (2008) 37: 372–377.
13. Chaudhary M, Bajaj S, Bohra S, Swastika N, Hande A. The domino effect: Role of hypoxia in malignant transformation of oral submucous fibrosis. Journal of Oral and Maxillofacial Pathology 2015;19:122-7.
14. Brennan P.A, Mackenzie N, Quintero M. Hypoxia-inducible factor 1alpha in oral cancer. J Oral Pathol Med 2005; 34: 385-389.

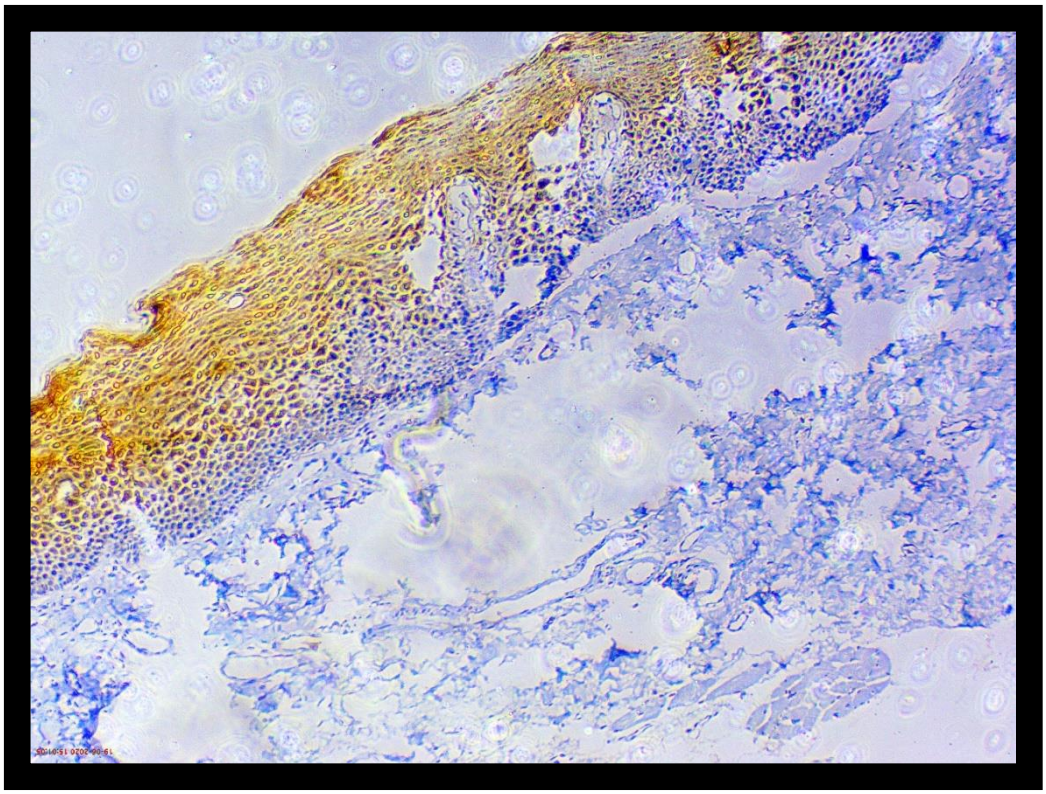

FIGURE1: IMMUNOHISTOCHEMICALLY STAINED SECTION OF OSMF SHOWING POSITIVE STAINING AT 10x

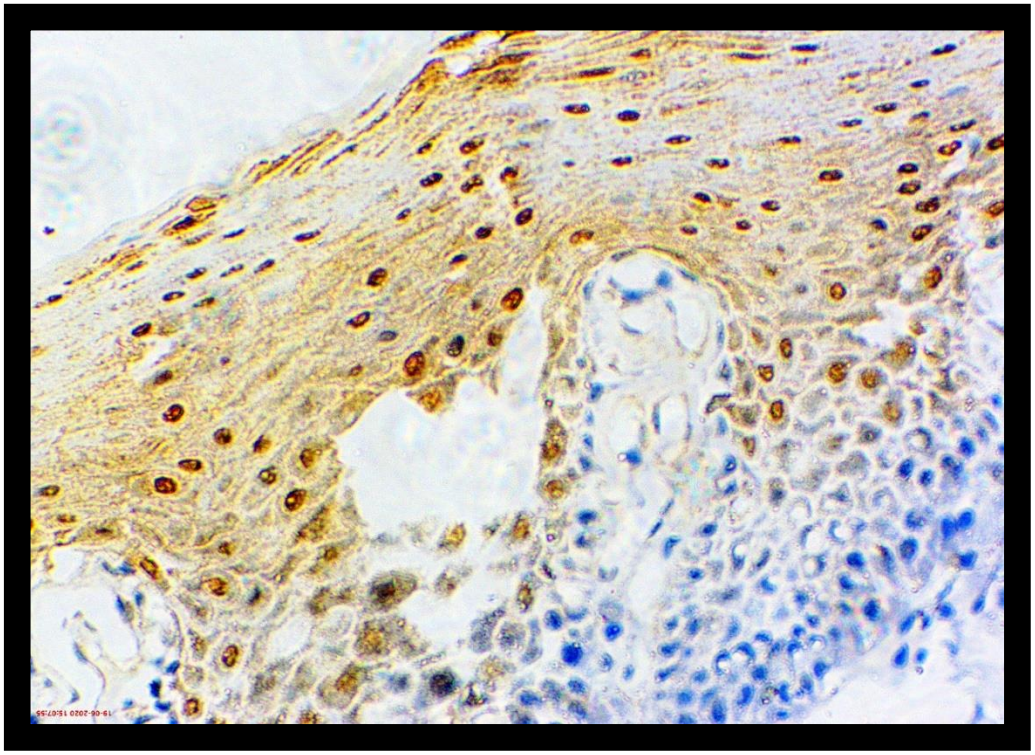

FIGURE 2a: IMMUNOHISTOCHEMICALLY STAINED SECTION OF OSMF SHOWING POSITIVE SATINING AT 40x

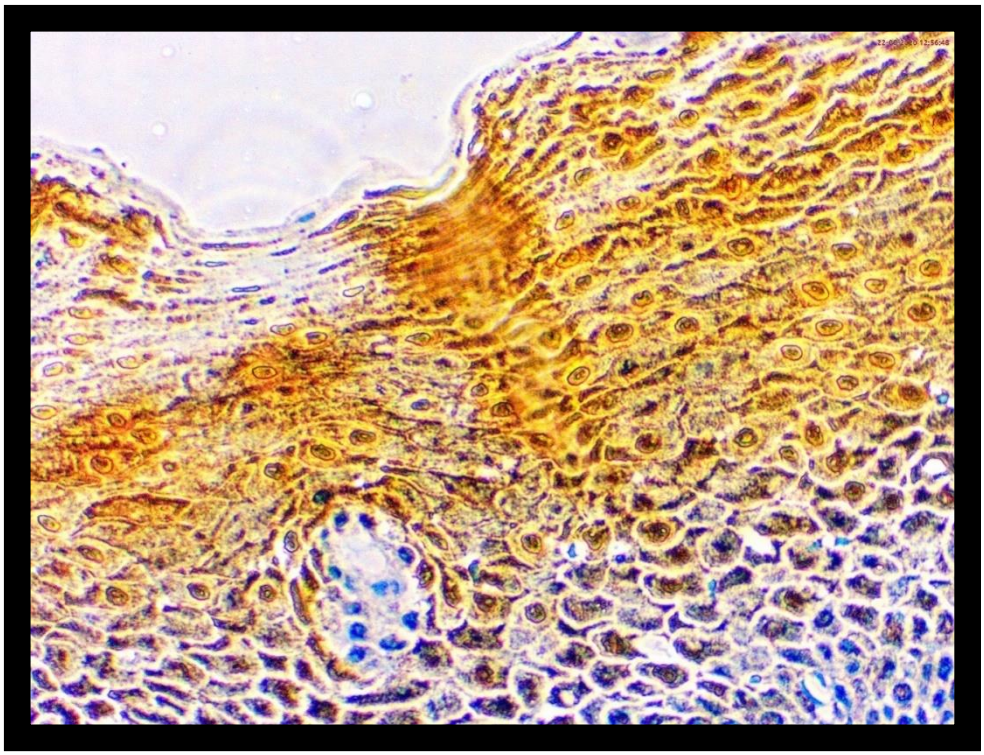

FIGURE 2b: IMMUNOHISTOCHEMICALLY STAINED SECTION OF OSMF SHOWING POSITIVE STAINING AT 40x

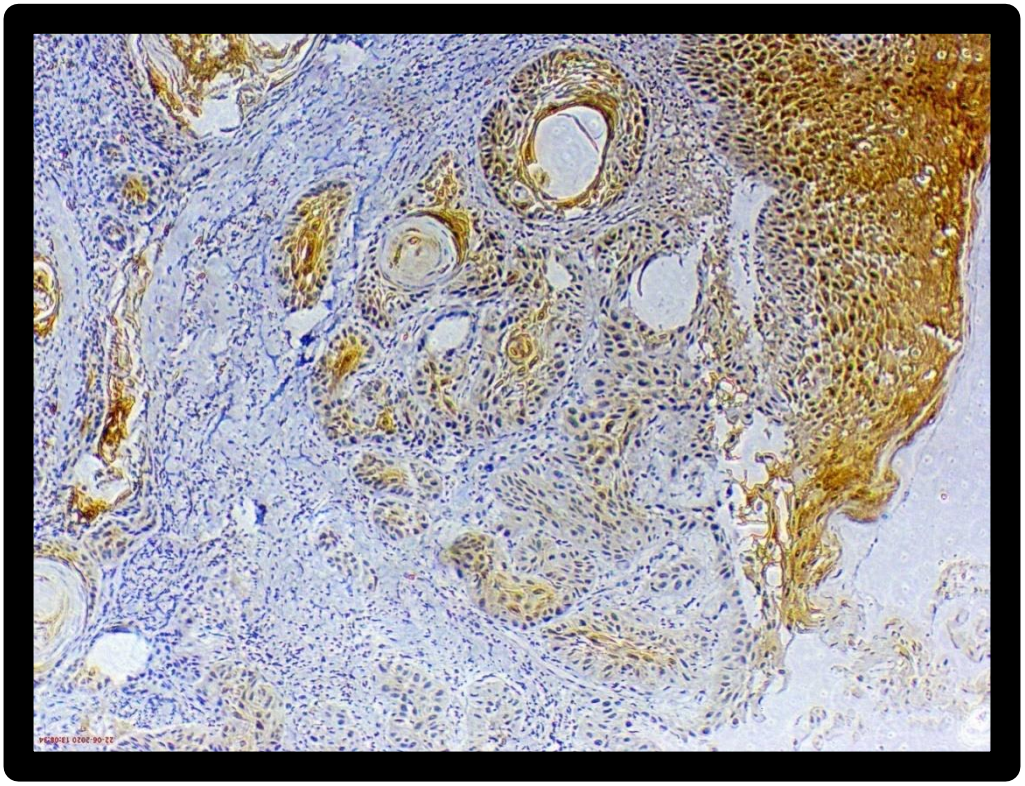

FIGURE 3: IMMUNOHISTOCHEMICALLY STAINED SECTION OF OSCC SHOWING POSITIVE STAINING AT 10x

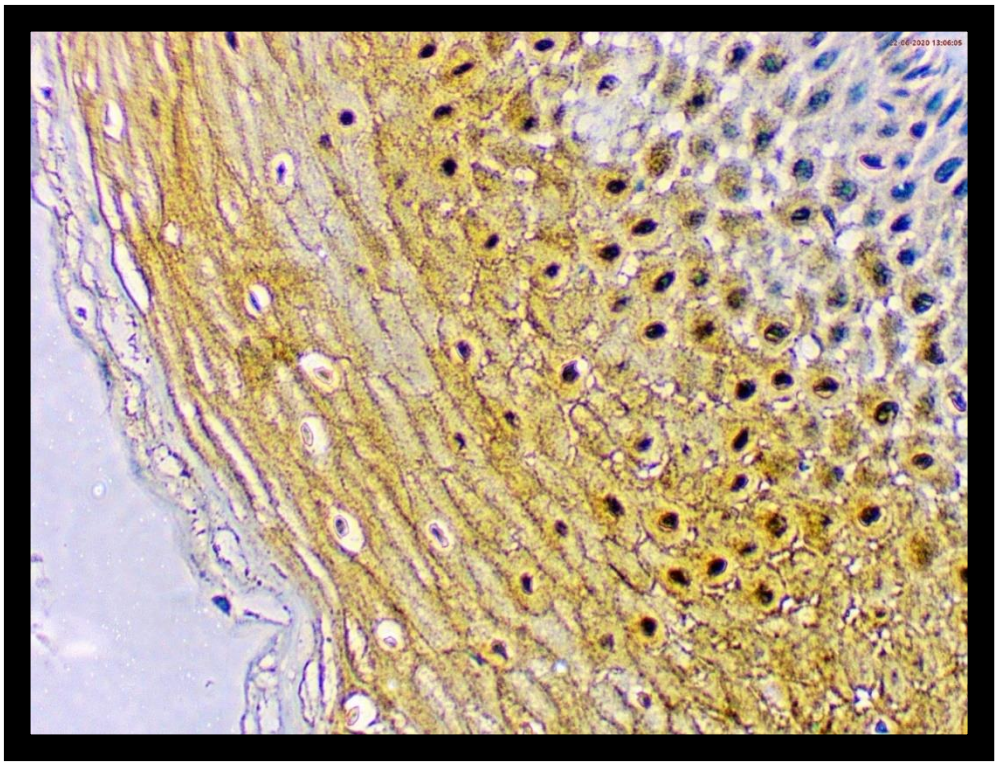

FIGURE 4: IMMUNOHISTOCHEMICALLY STAINED SECTION OF OSCC SHOWING POSITIVE STAINING AT 40 x

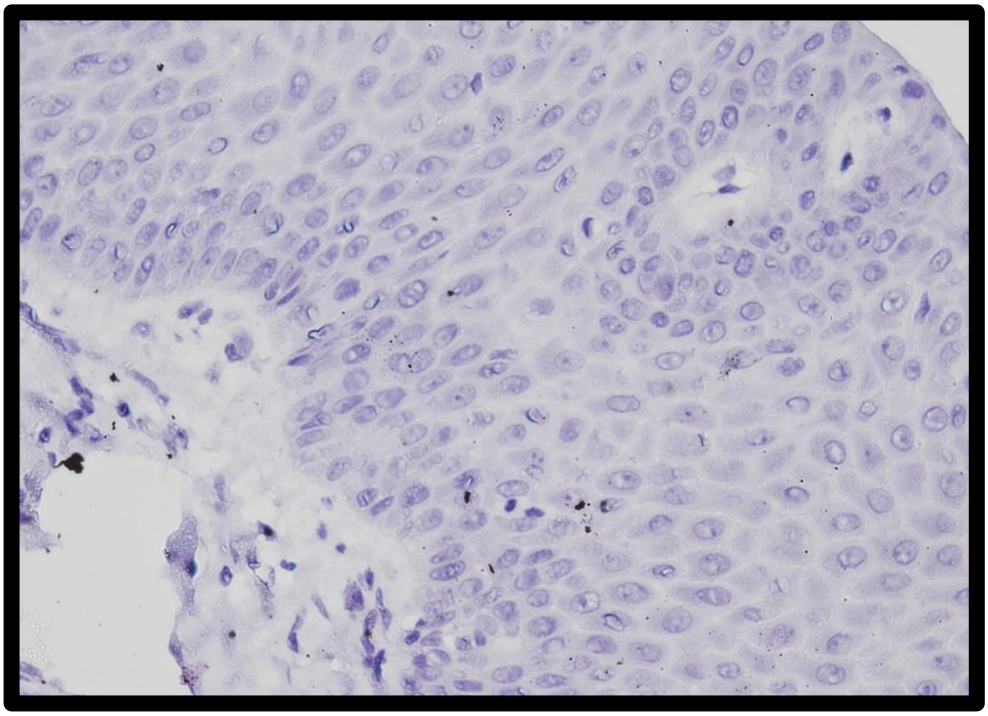

FIGURE 5: NORMAL ORAL MUCOSA SHOWING NO EXPRESSION OF HIF-1A