European Journal of Molecular & Clinical Medicine

ISSN 2515-8260 Volume 08, Issue 04, 2021

An Immunohistochemical assessment of Hypoxia Inducing Factor 1 alfa expression in Oral Epithelial Dysplasias And Oral Squamous Cell Carcinomas.

Dr.P.Ranga Raju, M.D.S. Dr. N. Govind raj Kumar, M.D.S., Dr. Nagaraj Anand, M.D.S. Dr. P. Ahalya M.D.S.

Senior Lecturer, Department of oral pathology, Vishnu Dental College, Bhimavaram.
Professor and HOD, Department of oral pathology, GSL Dental College, Rajamundary.
Professor and HOD, Department of oral pathology, Seema Dental College, Uttarakhand.
Senior Lecturer, Department of Pedodontics, Vishnu Dental College, Bhimavaram.

Abstract

HIF 1α is the intrinsic survival factor of dysplastic and tumour cells, to overcome the oxygen and nutrient deficits in the progression of OED and proliferation of OSCC. The entire spectrum of HIF 1α in OED and OSCC is not yet understood. The present study was done to evaluate the nature of expression of HIF 1α in OED and OSCC.

AIM:The study aims to evaluate the IHC expression of HIF 1α in OED and OSCC and also to investigate whether HIF 1α can be a predictive marker for malignant conversion of oral premalignant lesion.

OBJECTIVES:To analyse the IHC expression of HIF 1α quantitatively and qualitatively in OED and OSCC and to compare HIF 1α expression in OED and OSCC.

Materials and methods: Neutral buffered formalin fixed paraffin embedded blocks of 30 OED, 30 OSCC and 10 normal buccal mucosa cases that fall into WHO criteria were retrieved from the archives in the Department of Oral Pathology, Vishnu Dental College Bhimavaram. Clinical data was taken from the submitted biopsy forms. IHC staining of HIF1α was done according to the protocol. Quantitative, Qualitative and Semiquantitative assessment of the staining was done with the help of image J software and statistically analysed by SPSS software.

Results: Of the 30 stained HIF1α OED cases, 25 expressed HIF 1α positivity, of which 9, 7 and 9 were mild, moderate and severe OED respectively, accounting for 83.3% of staining.HIF 1α stained 30 OSCC cases, showed positivity in 22 cases, of which 7, 8 and 7 were Well, Moderately and Poorly Differentiated OSCC respectively, accounting for 73.3% of staining.

Conclusion: HIF1α a major contributor to tumor progression, is different in its IHC expression during different stages of OED and OSCC. Further studies could predict clinical outcome and survival of patients.

Key words: Oral Epithelial Dysplasia (OED), Oral Squamous Cell Carcinoma (OSCC),Hypoxia Inducing Factor-1 Alfa (HIF-1α).

Introduction: A sufficient resource of continuous oxygen perfusion is necessary for physiological development and as well as for pathological processes to ensue. Neoangiogenesis is considered as essential component for growth and progression of solid malignant tumours including those of the head and neck. This formation of new blood vessels is inadequate to provide enough oxygen to proliferating tumour cells, results in tissue hypoxia. HIF 1 is the intrinsic survival factor of tumour cells to overcome O_2 and nutrient deficits during proliferation and progression. HIF-1 induces various gene products which controls the energy, metabolism, neovascularization, survival, intracellular pH and cell migration, all these are the promoters of the growth of the tumour. Hence with these genetic alterations along with the intratumoral hypoxia, is strongly co related with the tumour grade, vascularity, metastasis, prognosis and overall survival.

OED is a combination of cytological and architectural changes due to abnormal and atypical cell proliferation which may ultimately progress to OSCC, the most prevalent malignant neoplasm of the oral cavity. A better understanding of the role of HIF 1α in regulation of these cellular processes is not only crucial for understanding “hypoxic tumour biology” and “epithelial dysplasia’s” of the oral cavity but also for developing

the novel anticancer therapies. In this dissertation the expression of HIF is summarized in the OSCC and the OED.

Observations and Results: The study sample included 70 cases of which 10 Mild OED,10 Moderate OED,10 Severe OED,10 Well differentiated OSCC,10 Moderately differentiated OSCC,10 Poorly differentiated OSCC, and 10 normal healthy buccal mucosa without any adverse habits as controls.

Demographic and Clinical data has been collected from the requisition forms sent along with the specimen. For four cases of poorly differentiated OSCC adequate details have not been mentioned which were labelled as not specified since the data could not be gathered.

DEMOGRAPHIC AND CLINICAL DETAILS OF THE CASES:

Gender	**Male (n=25)**			**Female (n=5)**		
Age (years)	**MILD DYSPLASIA**	**MODERATE DYSPLASIA**	**SEVERE DYSPLASIA**	**MILD DYSPLASIA**	**MODERATE DYSPLASIA**	**SEVERE DYSPLASIA**
1 to 10	0	0	0	0	0	0
11 to 20	1	0	0	0	0	0
21 to 30	2	1	0	0	0	0
31 to 40	0	1	1	0	1	0
41 to 50	0	2	4	0	0	0
51 to 60	6	2	0	0	1	2
61 to 70	1	1	0	0	0	1
>70	0	1	2	0	0	0

Table 1: Age and gender distribution of oral epithelial dysplasia.

Table 2: Age and gender distribution of oral squamous cell carcinomas.

Gender	**Male (n=16)**			**Female (n=14)**		
Age (years)	**WDSCC**	**MDSCC**	**PDSCC**	**WDSCC**	**MDSCC**	**PDSCC**
1 to 10	0	0	0	0	0	0
11 to 20	0	0	0	0	0	0
21 to 30	0	0	1	0	0	0
31 to 40	0	0	0	1	2	0
41 to 50	0	0	0	2	1	0
51 to 60	1	1	4	1	2	1
61 to 70	2	1	1	1	1	0
>70	2	0	0	0	1	0
Not Specified	0	0	3	0	0	1

Of the 30 Dysplasia cases 25 were Male and 5 were Female and thus with male to female predilection of 3:1. The peak age of incidence for Dysplasia cases was observed in 6th decade with an average age of incidence at 49.53 years (range: 17-75 years). The clinical data of the four cases of poorly differentiated squamous cell carcinoma couldn't be gathered and among the 26 cases of oral squamous cell carcinomas data obtained showed equal male to female predilection. The peak age of incidence for squamous cell carcinoma cases similar to dysplasia was observed in 6th decade with an average age of incidence at 48.6 years (range: 25-75 years).A total of 10 cases of normal healthy gingiva without any deleterious habits preferably from retro molar region during impactions was taken as a control group with an average age of 41 years in which 4 were females and 6 were males.

Table 3.Site of occurrence of oral epithelial dysplasia

Cases	Buccal mucosa	Palate	Floor of the mouth	Tongue	Lip
Mild dysplasia	6	1	0	3	0
Moderate dysplasia	6	2	1	1	0
Severe dysplasia	7	0	0	2	1

Table 4.Site of occurrence of oral squamous cell carcinoma

Cases	Buccal mucosa	Palate	Floor of the mouth	Tongue	Lip
WDSCC	7	0	1	1	0
MDSCC	6	1	1	1	1
PDSCC	4	0	0	1	1

Pin-Yi Lin study found a higher mean nuclear HIF-1α in OSCCs in drinkers and smokers than in OSCCs in non-drinkers and non-smokers. Li et al. (84) showed a significantly higher expression of mRNA in rat fed with excessive amount of ethanol than in controls. Szabo et al. (85) reported that alcohol injury induces a high expression of HIF-1α in gastric mucosa. Ethanol is also found to increase the expression of HIF-1α protein[96]. Therefore, the high expression of HIF-1α in OSCCs may be due to the direct stimulation of cancer cells by alcohol present in the patients' blood. The long-term cigarette smoking increases the incidence of ischemic vascular disease and atherosclerosis; this result may attribute to the cigarette smoke inhibition of VEGF expression and angiogenesis in hypoxic condition[108]. In OSCC patients with smoking habit, long-term cigarette smoking may cause insufficient vascular supply to the OSCC and subsequent formation of some hypoxic tumor areas, which in turn may result in a high HIF-1α expression in OSCCs[26].The Habits associated with Oral Epithelial Dysplasia and Oral Squamous Cell Carcinomas in the present study reported are as follows

Table 5.Dysplasia and their distribution with respect to habits.

Oral epithelial dysplasia	Smoking	No Smoking	Smoking and Alcohol	Alcohol
Mild dysplasia	5	2	3	0
Moderate dysplasia	8	1	1	0
Severe dysplasia	3	0	7	0

Table No.6 groups * Habits Cross tabulation

			HABITS			Total
			Smoking	No Smoking	Smoking and	
Groups	Mild Dysplasia	Count	5	2	3	10
		% within groups	50.0%	20.0%	30.0%	100.0%
		% within HABITS	33.3%	50.0%	27.3%	33.3%
		% of Total	16.7%	6.7%	10.0%	33.3%
	Moderate Dysplasia	Count	8	1	1	10
		% within groups	80.0%	10.0%	10.0%	100.0%

		% within HABITS	53.3%	25.0%	9.1%	33.3%
		% of Total	26.7%	3.3%	3.3%	33.3%
	Severe Dysplasia	Count	2	1	7	10
		% within groups	20.0%	10.0%	70.0%	100.0%
		% within HABITS	13.3%	25.0%	63.6%	33.3%
		% of Total	6.7%	3.3%	23.3%	33.3%
Total		Count	15	4	11	30
		% within groups	50.0%	13.3%	36.7%	100.0%
		% within HABITS	100.0%	100.0%	100.0%	100.0%
		% of Total	50.0%	13.3%	36.7%	100.0%

Table 7.Squamous cell carcinoma and distribution with respect to habits.

OSCC	Smoking	No smoking	Smoking and Alcohol	Alcohol
WDSCC	5	1	3	1
MDSCC	3	1	5	1
PDSCC	2	1	3	0

Table 8.groups STAINING Cross tabulation

			STAINING DOS			Total
			1.00	2.00	3.00	
Groups	Mild Dysplasia	Count	1	7	2	10
		% within groups	10.0%	70.0%	20.0%	100.0%
		% within STAINING DOS	20.0%	43.8%	22.2%	33.3%
		% of Total	3.3%	23.3%	6.7%	33.3%
	Moderate Dysplasia	Count	3	7	0	10
		% within groups	30.0%	70.0%	0.0%	100.0%
		% within STAINING DOS	60.0%	43.8%	0.0%	33.3%
		% of Total	10.0%	23.3%	0.0%	33.3%
	Severe Dysplasia	Count	1	2	7	10
		% within groups	10.0%	20.0%	70.0%	100.0%
		% within STAINING DOS	20.0%	12.5%	77.8%	33.3%
		% of Total	3.3%	6.7%	23.3%	33.3%
Total		Count	5	16	9	30
		% within groups	16.7%	53.3%	30.0%	100.0%
		% within STAINING DOS	100.0%	100.0%	100.0%	100.0%
		% of Total	16.7%	53.3%	30.0%	100.0%

Conventional hematoxylin and eosin staining (H&E): Sections were done for the presence of architectural and cytological changes which are generally referred to as epithelial dysplasia. Inter and intra observer variations in the grading of the lesions was noted to avoid subjective bias and graded as mild, moderate and severe dysplasias.

Correlation of antigen reactivity to the histopathological findings

Normal oral mucosa (NOM): The nuclear HIF-1α staining in the normal epithelium was found predominantly in the spinous cells and rarely in the basal and superficial cells, while the cytoplasmic HIF-1α staining was expressed faintly in the basal cells and intense expression in the superficial cells.

HIF 1α in the OED and OSCC: The 30 Dysplastic cases stained for HIF-1α showed positivity for 25 cases (83.3%) of staining in the study group, of which 9 were mild dysplasia, 7 were moderate dysplasia and 9 were severe dysplasia cases. The location of HIF-1α showed a progressive switch from cytoplasm in mild epithelial dysplasia to nuclear and cytoplasm in moderate epithelial dysplasia to nuclear in the severe dysplasia. Faint expression of cytoplasmic was seen in few cases of severe dysplasia.

The staining intensity was gradually raised from Mild OED>Moderate OED>Severe OED and from well differentiated OSCC>moderate differentiated OSCC>poorly differentiated OSCC with RBCs as the internal control. One way ANOVA statistical analysis showed statistical significance in mild and moderate intensity of staining with p=0.015 and p=0.001 respectively but in intense intensity of staining was statistically insignificant with p=0.469.

Expression of HIF 1α in different strata of the epithelium of OED : HIF-1α variable expressivity in the different layers of the dysplastic epithelium like Mild dysplasia cases showing the expression in the upper layer of the stratum spinosum and basal cell layers, Moderate Dysplasia exhibiting all along the stratum spinosum and severe dysplasia cases showing no expression of HIF-1α in the basal and para basal layers as well as in the stratum superficial but restricting its expression to the stratum spinosum. (Fig.3 to Fig.12).One moderate epithelial dysplasia case expressed HIF-1α in the upper layers of stratum spinosum which was very faint. HIF 1α was very less in its expression in the moderate epithelial dysplasia than the mild and severe dysplasia cases.

The two severe epithelial dysplasia cases showing no expression of HIF-1α in the basal and parabasal layers as well as in the stratum superficial of the severe epithelial dysplasia cases (fig.5-8).The two severe epithelial dysplasia cases depicted in the pictures reveal that within the severe epithelial grading one shows nuclear expression and the other shows the cytoplasmic expression.

Expression of HIF 1α in various grades of OSCC: Of the 30 cases of OSCC stained for HIF-1α revealed its variable shuttling nature between the cytoplasm and nucleus in the 22 HIF-1α positive cases accounting for around 73.3% of staining of the OSCC in the study. The HIF-1α was not expressed in the 8 OSCC of which three were poorly differentiated OSCC cases, two were moderately differentiated OSCC cases and 3 were well differentiated OSCC.

Well differentiated OSCC: HIF-1α immunoreactivity appeared as a localized or focal granular cytoplasmic and/or nuclear brown staining of mainly the peripheral malignant cell layers in the keratin and the epithelial pearls. HIF-1α was more pronounced around the areas of keratinization and necrosis, as depicted in figure 17. In most cases, HIF-1α appeared more intense in areas away from the blood vessels. Inflammatory cells showed nuclear staining more intensely than the cytoplasmic staining. Cytoplasmic HIF-1α was predominant than the nuclear staining or may appear the only expression pattern in the 7 cases stained for HIF-1α. Staining pattern was predominantly evident at the periphery of the tumor islands and absent in the central keratin pearls.

Moderately differentiated OSCC: Of the 10 cases four cases showed localized HIF-1α immunoreactivity either nuclear or cytoplasmic while four cases showed diffuse HIF-1α throughout the entire tumor which was independent of vessel proximity. Only two cases were not positive for the HIF-1α.Nuclear HIF1α was predominant than cytoplasmic staining in the stained 8 cases.

Poorly differentiated OSCC: HIF-1α expression in most cases of poorly differentiated OSCC (7 out of 10cases) exhibited intense 5 focal staining and as the intense diffuse nuclear reaction of the invading malignant cells, with or without cytoplasmic localization. Only one case showed cytoplasmic HIF-1α reactivity without nuclear pattern of expression. Staining pattern was predominantly evident at the center of the tumor islands and absent in the periphery which is opposite to that of the staining what was appreciated in the well differentiated OSCC.

Table No.9

ANOVA of mild, moderate and severe staining intensity of HIF 1α

		Sum of Squares	Df	Mean Square	F	Sig.
mild	Between Groups	13412.69	6	2235.448	2.871	0.015
	Within Groups	49056.3	63	778.671		
	Total	62468.99	69			
moderate	Between Groups	6634.343	6	1105.724	4.275	0.001
	Within Groups	16295.6	63	258.66		
	Total	22929.94	69			
severe	Between Groups	3120.743	6	520.124	0.946	0.469
	Within Groups	34627.1	63	549.637		
	Total	37747.84	69			

Table no.10

ANOVA of HIF 1α expression of Cytoplasmic, Nuclear and Cytoplasmic and Nucleus staining

		Sum of Squares	df	Mean Square	F	Sig.
nuclear	Between Groups	200759.143	6	33459.86	3.458	0.005
	Within Groups	609580.3	63	9675.878		
	Total	810339.443	69			
cytoplasmic	Between Groups	327331.286	6	54555.21	2.387	0.038
	Within Groups	1439841.8	63	22854.63		
	Total	1767173.086	69			
Nuclear & cytoplasmic	Between Groups	119050.686	6	19841.78	0.906	0.496
	Within Groups	1379124.3	63	21890.86		
	Total	1498174.986	69			

Semi quantitative Analysis of HIF 1α in OED and OSCC

Here are the Semiquantitative scoring criteria obtained for both OED and OSCC by adding the % of positive cells and points for expression.

ISSN 2515-8260 Volume 08, Issue 04, 2021

Scoring Index for Mild Dysplasia				
CaseNo.	Pattern	Intensity	Cell %	Overall Score
1	Diffuse	2	3	5
2	Local	1	1	2
3	Local	1	1	2
4	Local	1	1	2
5	Local	1	1	2
6	Local	1	1	2
7	Diffuse	3	4	7
8	Local	1	1	2
9	Local	1	1	2
10	No staining	0	0	0

Scoring Index for Moderate Dysplasia				
Case No.	Pattern	Intensity	Cell %	Overall Score
1	Local	3	1	4
2	Local	1	1	2
3	Local	1	1	2
4	Local	1	1	2
5	Local	2	1	3
6	No staining	0	0	0
7	No staining	0	0	0
8	Local	1	1	2
9	Local	1	1	2
10	No staining	0	0	0

Scoring Index for Severe Dysplasia				
Case No	Pattern	Intensity	Cell %	Overall Score
1	Local	3	1	4
2	Diffuse	3	3	6
3	Diffuse	3	3	6
4	Diffuse	3	3	6
5	Diffuse	3	3	6
6	Local	2	2	4
7	Diffuse	3	4	7
8	Diffuse	2	3	5
9	Diffuse	3	4	7
10	No staining	0	0	0

ISSN 2515-8260 Volume 08, Issue 04, 2021

Scoring Index for Well Differentiated OSCC				
Case No.	Pattern	Intensity	Cell %	Overall Score
1	Focal	1	1	2
2	Diffuse	1	4	5
3	Focal	1	1	2
4	Focal	1	1	2
5	Focal	1	1	2
6	No staining	0	0	Nil
7	Focal	1	1	2
8	Focal	2	1	3
9	Focal	3	2	5
10	No staining	0	0	Nil

Scoring Index for Moderately differentiated OSCC				
Case No	Pattern	Intensity	Cell %	Overall Score
1	Diffuse	2	3	5
2	Focal	1	1	2
3	Focal	1	1	2
4	Focal	1	1	2
5	Diffuse	2	4	6
6	No staining	0	0	0
7	No staining	0	0	0
8	Focal	0	0	0
9	Diffuse	2	4	6
10	Diffuse	2	4	6

Scoring Index for Poorly differentiated OSCC				
Case No.	Pattern	Intensity	Cell %	Overall Score
1	Diffuse	2	3	5
2	Focal	3	2	5
3	Focal	3	1	4
4	Focal	1	1	2
5	Diffuse	2	3	5
6	No staining	0	0	0
7	Focal	3	2	5
8	Focal	3	2	5
9	No staining	0	0	0
10	No staining	0	0	0

Table No.9

Semiquantitative scoring criteria of OED

Overall Score		Mild dysplasia	Moderate dysplasia	Severe dysplasia
Negative	≤10% of cells stained +ve	1	3	1
2-3 Points	Weak Expression	7	6	0
4-5 Points	Moderate Expression	1	1	3
6-7 Points	Strong Expression	1	0	6

Table No.10 Semiquantitative scoring criteria of OSCC

Overall Score		PDSCC	MDSCC	WDSCC
Negative	≤10% of cells stained +ve	3	3	2
2-3 Points	Weak Expression	1	3	6
4-5 Points	Moderate Expression	6	4	2
6-7 Points	Strong Expression	0	0	0

Table No.11 Overall Staining of OSCC

Cases	Total Unstained	Total stained	Diffuse	Focal
PDSCC	3	7	2	5
MDSCC	2	8	4	4
WDSCC	3	7	1	6
Total	8	22	7	15

Table No.12 Overall staining of OED

Cases	Total Unstained	Total stained	Diffuse	Focal
Mild dysplasia	1	9	2	7
Moderate dysplasia	3	7	0	7
Severe dysplasia	1	9	7	2
Total	5	25	9	16

In the present study overall semi quantitative score showed strong staining score of 7 of which all the seven were OED. Moderate staining score of 19 of which 9OED cases and 10 OSCC cases. Weak staining score of 23 of which the 13 OED cases and 10 OSCC cases. No staining score of 13 of which 5 are OED and 8 are OSCC. Hence the value obtained by this scoring criteria showed highest score of weak staining around 28.

Well differentiated OSCC: According to the Scoring criteria of the 10 cases, 3 revealed no staining, 6 revealed weak intensity of staining and 1 revealed strong intensity of staining. Of the stained 7 cases one exhibited diffuse staining and the other six exhibited focal staining of the HIF 1α.

ISSN 2515-8260 Volume 08, Issue 04, 2021

The percentage of staining of the two groups by semi quantitative analysis with a similar method followed by Peter Birner et al and Barbara Bachtiary et al, is as follows

Table No.13

Intensity	OSCC	OED
Weak	33.30%	43.30%
Moderate	40%	16.70%
Strong	0	23.30%
No staining	26.70%	16.70%

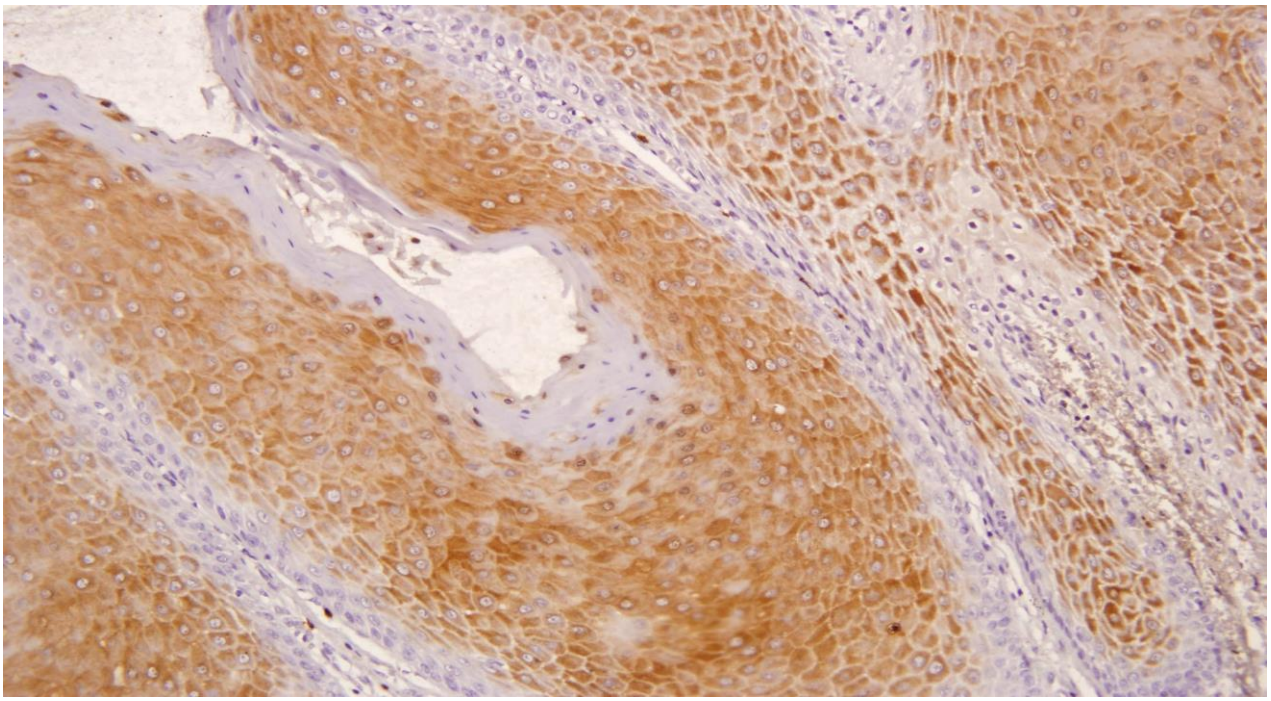

Figure 3: Cytoplasmic expression of HIF 1α without the nuclear expression in the stratum spinosum of the mild epithelial dysplasia (20x Magnification.)

ISSN 2515-8260 Volume 08, Issue 04, 2021

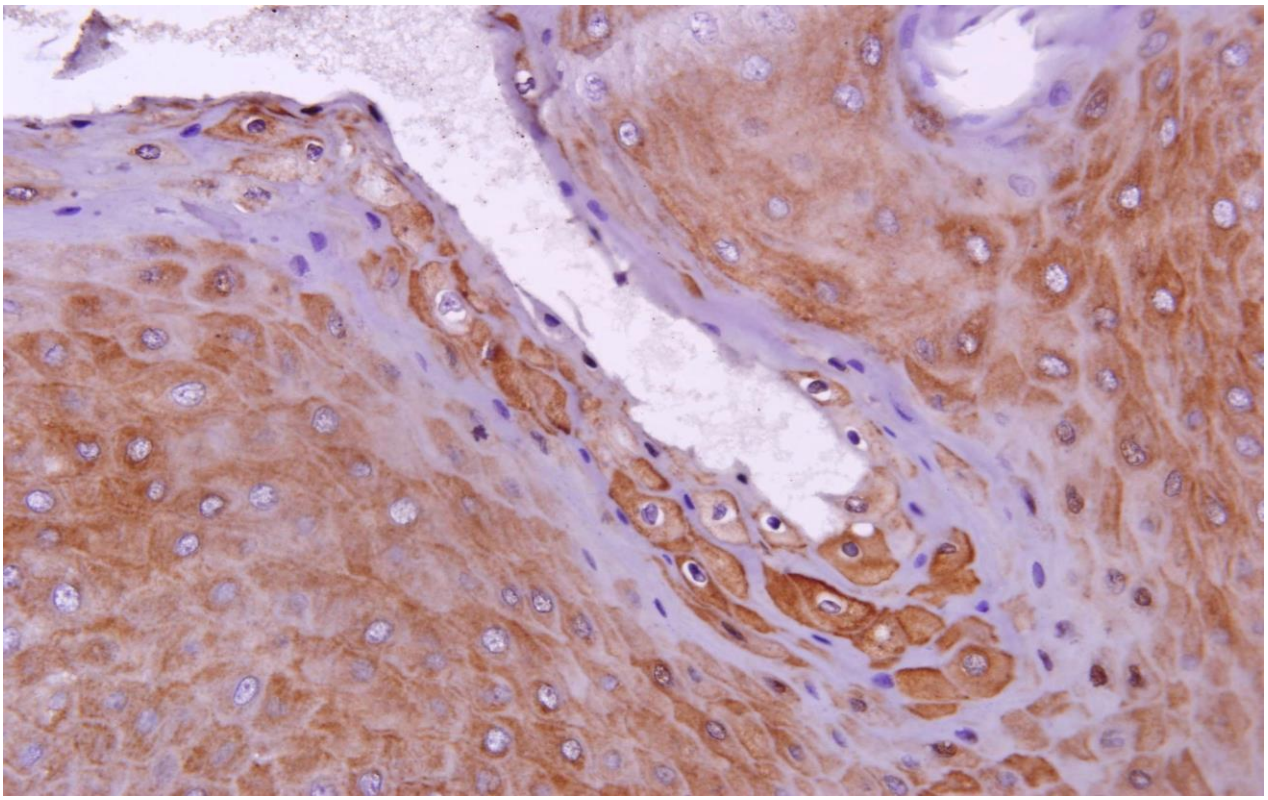

Figure 4: Cytoplasmic expression of HIF 1α without the nuclear expression in the mild epithelial dysplasia (40x Magnification.)

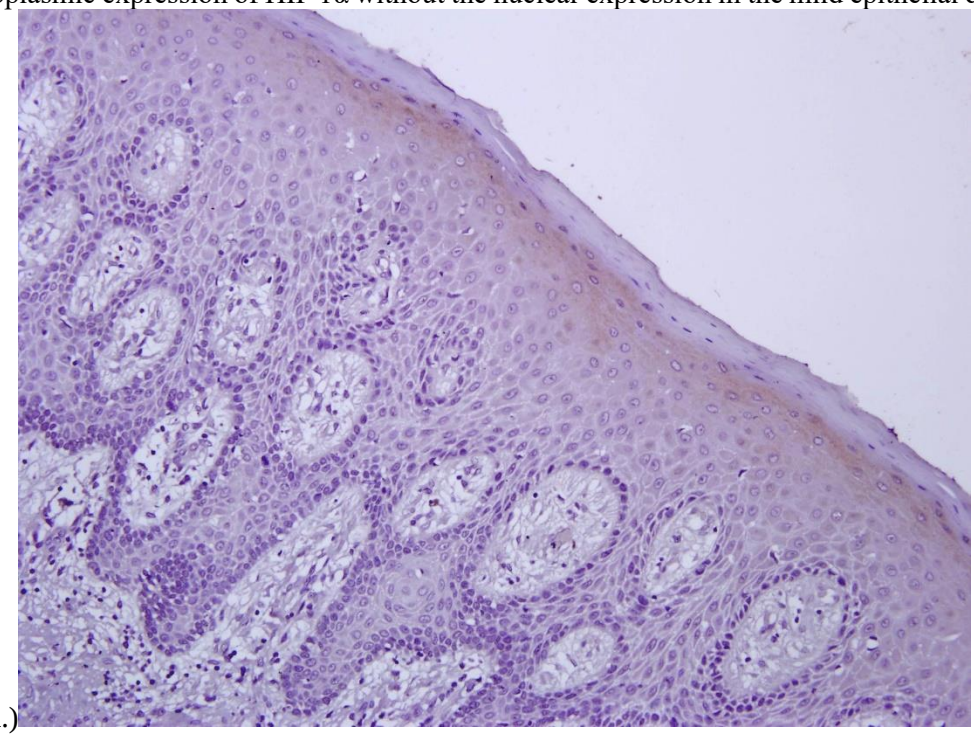

Figure 5: HIF 1 α cytoplasmic expression without the nuclear expression of the superficial region of the stratum spinosum in the mild epithelial dysplasia (40x Magnification.)

ISSN 2515-8260 Volume 08, Issue 04, 2021

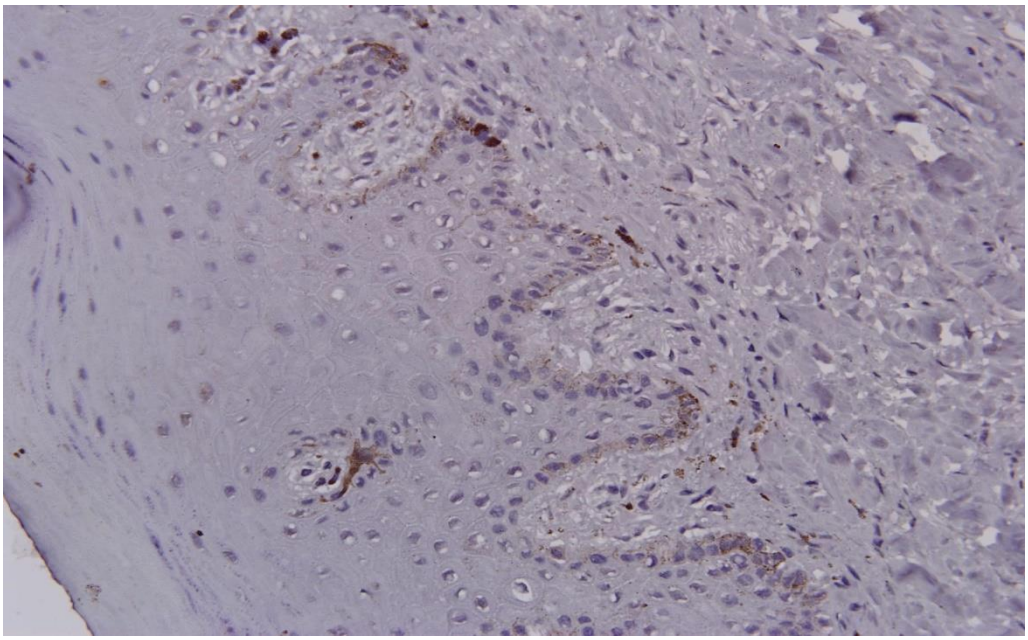

Figure 6: HIF 1α Mild cytoplasmic expression of the basal cells without the nuclear expression in the mild epithelial dysplasia (40x Magnification.)

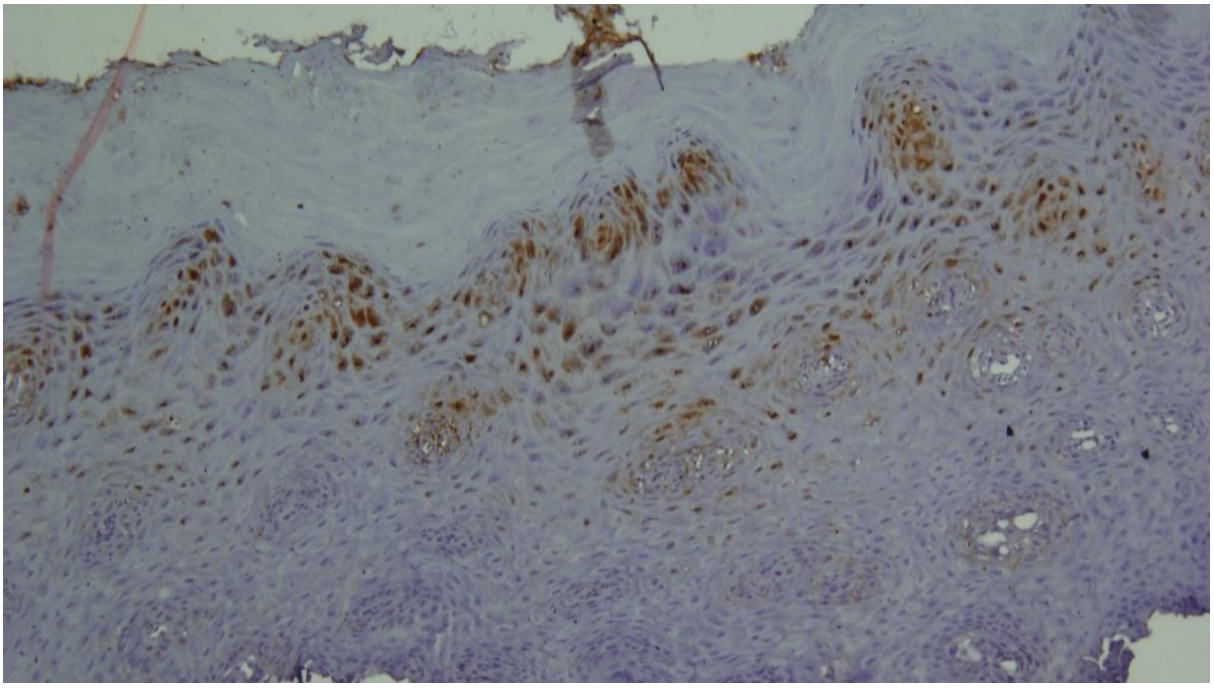

Figure 7: Expression of HIF 1α in the stratum spinosum of the moderate epithelial dysplasia (10x Magnification.)

ISSN 2515-8260 Volume 08, Issue 04, 2021

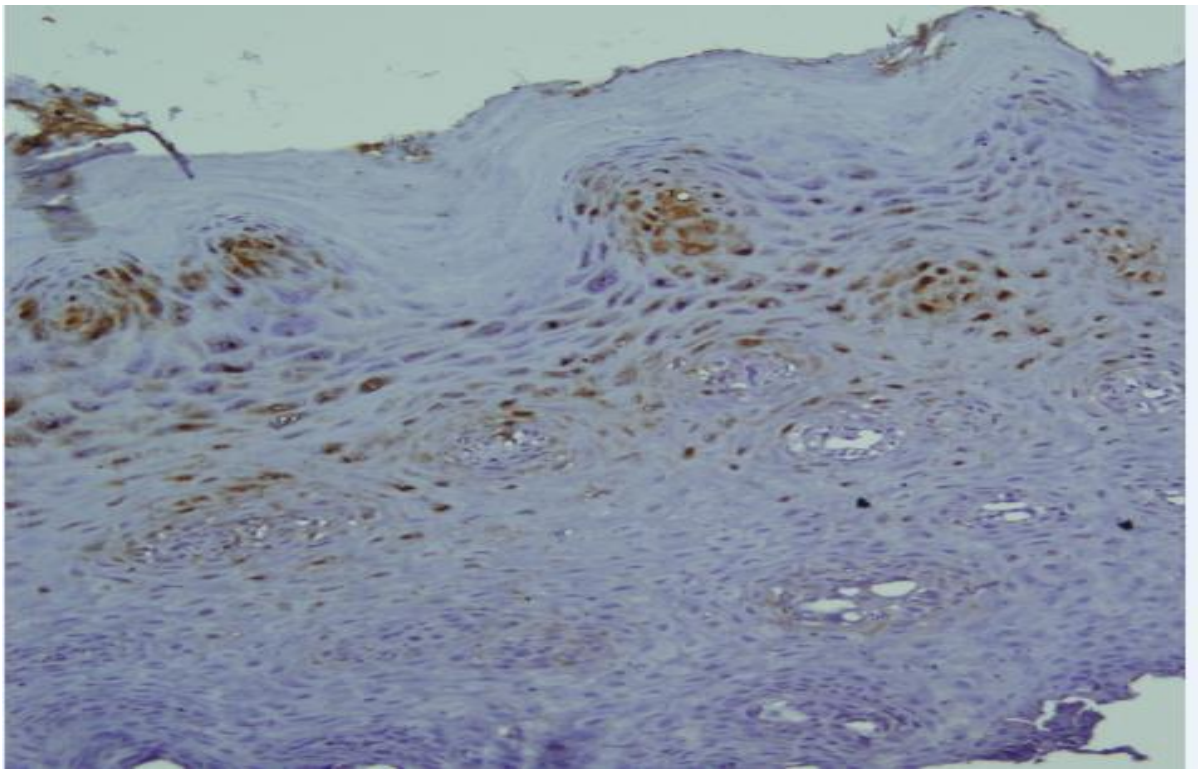

Figure 8: Expression of HIF 1α in the stratum spinosum of the moderate epithelial dysplasia (20x Magnification.)

ISSN 2515-8260 Volume 08, Issue 04, 2021

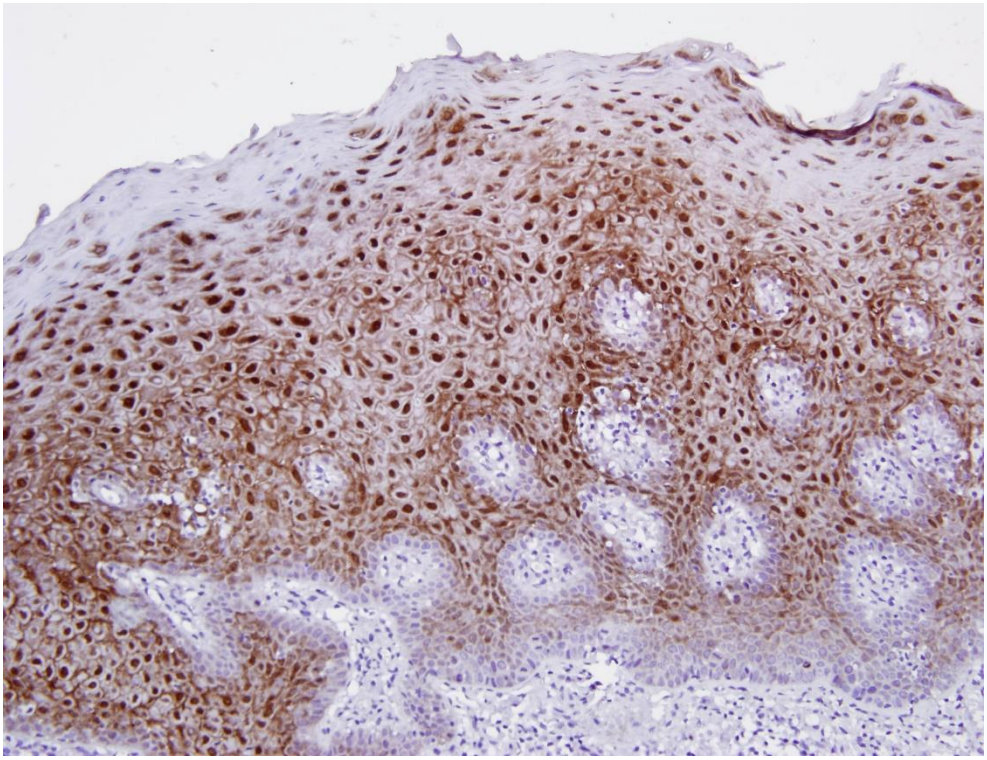

Figure 9: A Severe epithelial dysplasia case in which HIF 1α expression in the basal and para basal layers of the epithelium was negligible with a faint expression. While in the stratum superficial of the epithelium there was not HIF 1alpha expression as appreciable as in the stratum spinosum. (20x magnification).

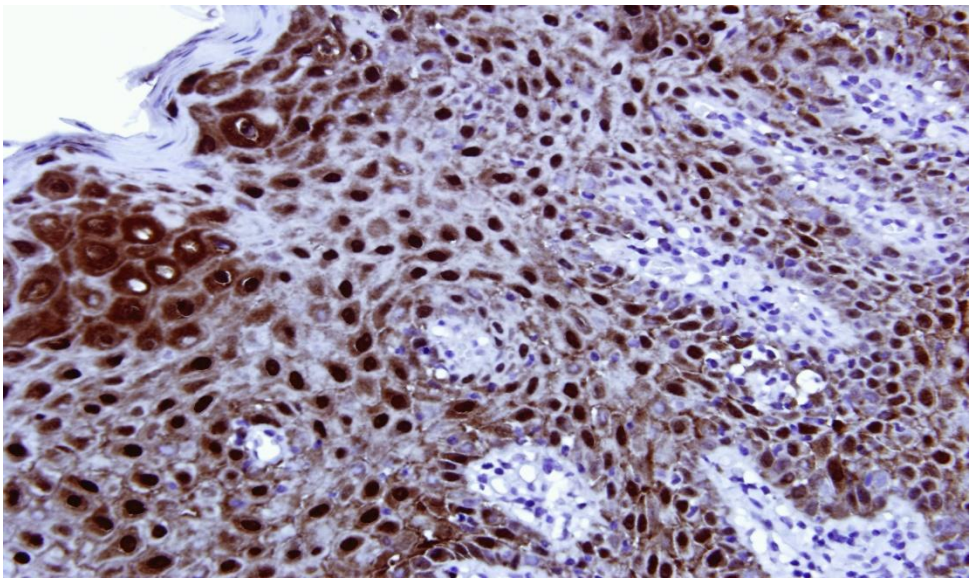

Figure 10: A Severe epithelial dysplasia case where HIF 1α expression is more predominant in the stratum spinosum under 40x Magnification

ISSN 2515-8260 Volume 08, Issue 04, 2021

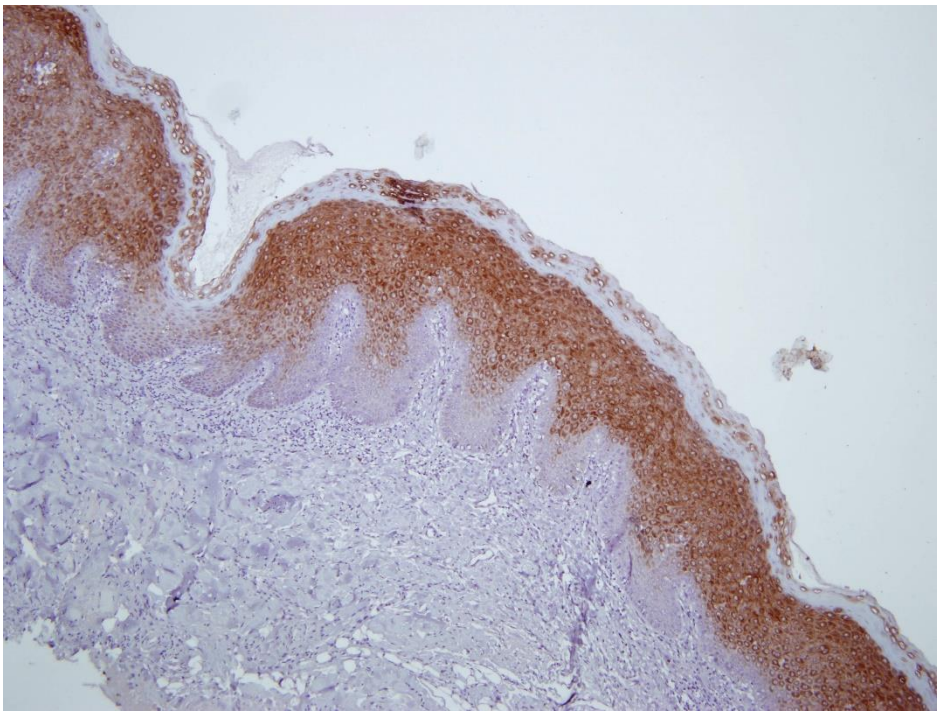

Figure 11: A Severe epithelial dysplasia case in which HIF 1α expression in the basal and para basal layers of the epithelium was negligible with a faint expression. While in the stratum superficial of the epithelium there was not HIF 1α expression as appreciable as in the stratum spinosum. (20x magnification).

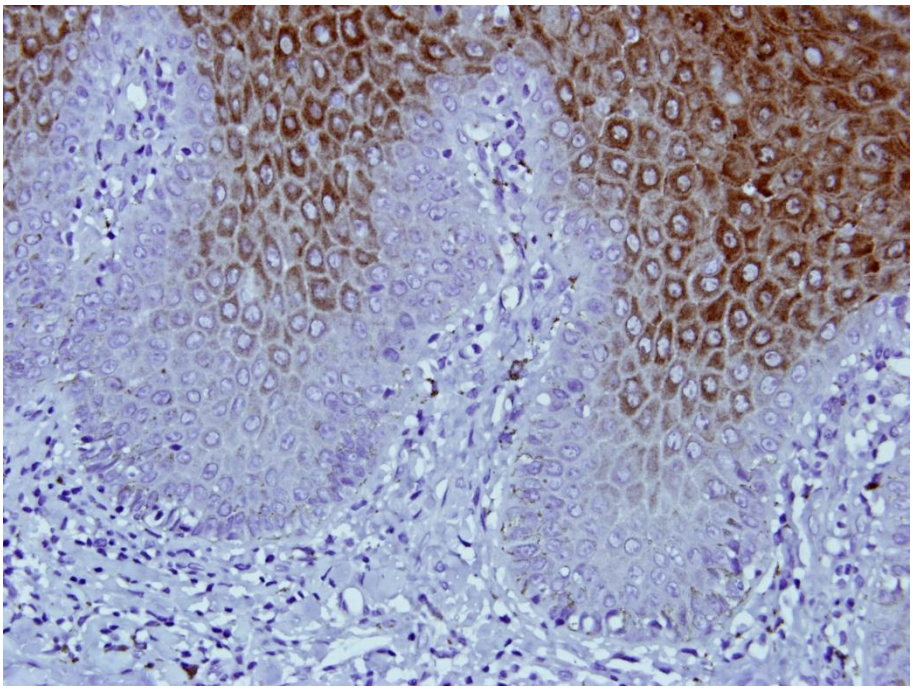

Figure 12: A Severe epithelial dysplasia case in which HIF 1α expression in the basal and para basal layers of the epithelium was negligible with a faint expression. (40x Magnification)

ISSN 2515-8260 Volume 08, Issue 04, 2021

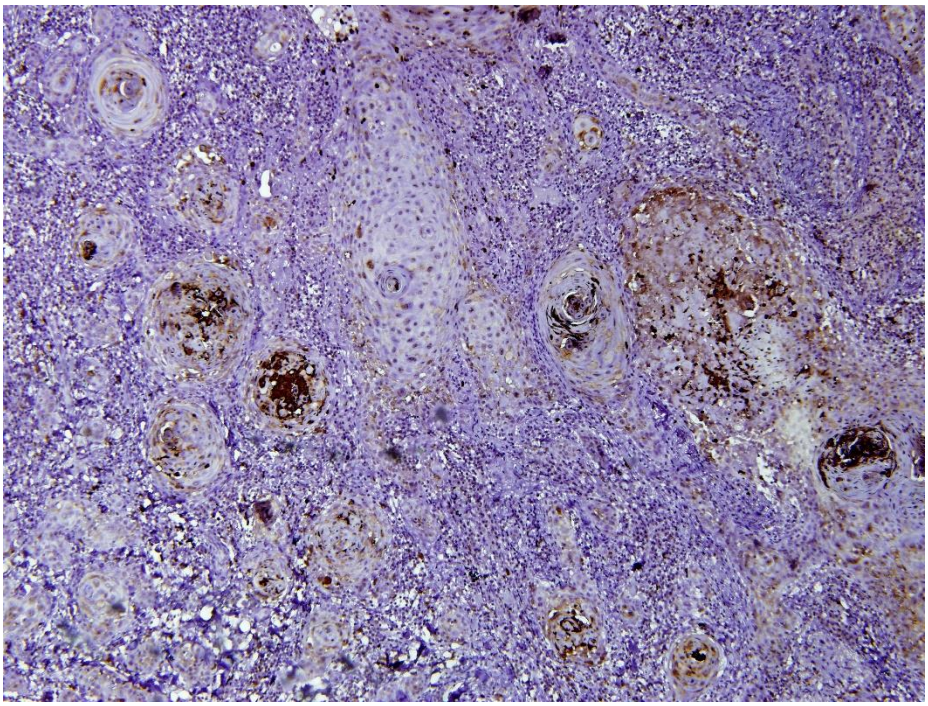

Figure 13: Mild HIF 1 alpha cytoplasmic expression of the tumor cells without the nuclear expression in the well differentiated squamous cell carcinoma. HIF 1α expressed in areas of necrosis and keratinization. (10x Magnification.)

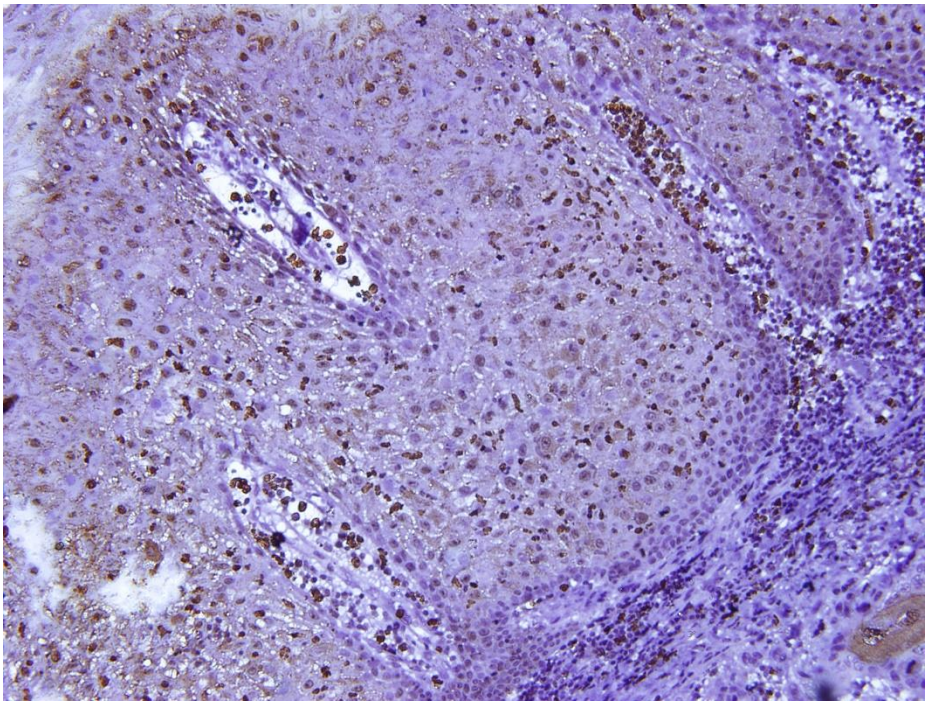

Figure 14: HIF 1α cytoplasmic expression of the tumor cells without the nuclear expression, Nuclear expression without the cytoplasmic expression and both nuclear and cytoplasmic expression in the moderately differentiated squamous cell carcinoma (20x Magnification.)

ISSN 2515-8260 Volume 08, Issue 04, 2021

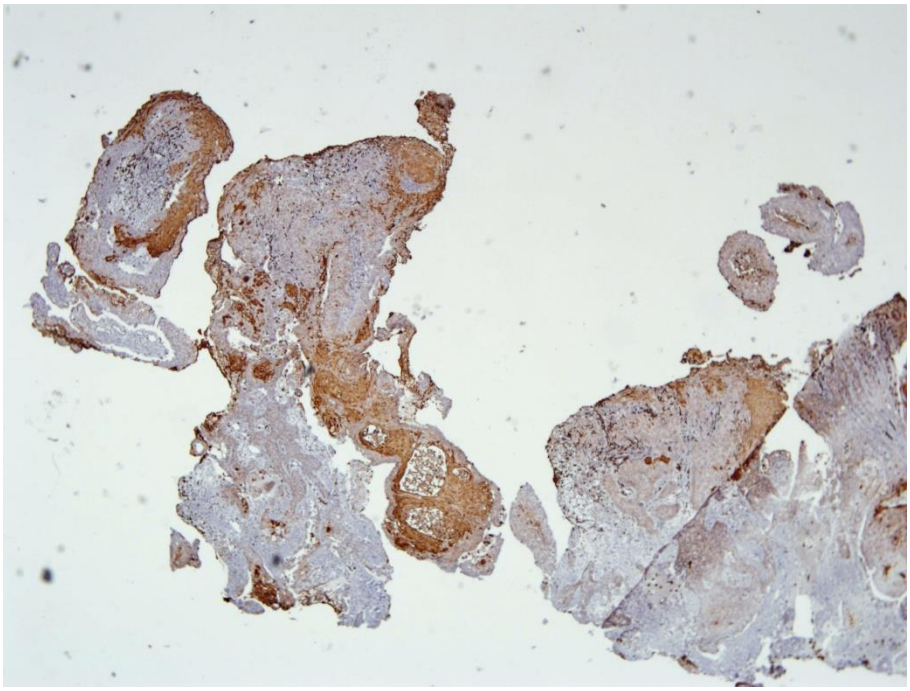

Figure 15: Variable expression of HIF 1α in the intensity of the tumor islands staining in the poorly differentiated squamous cell carcinoma (4x Magnification.)

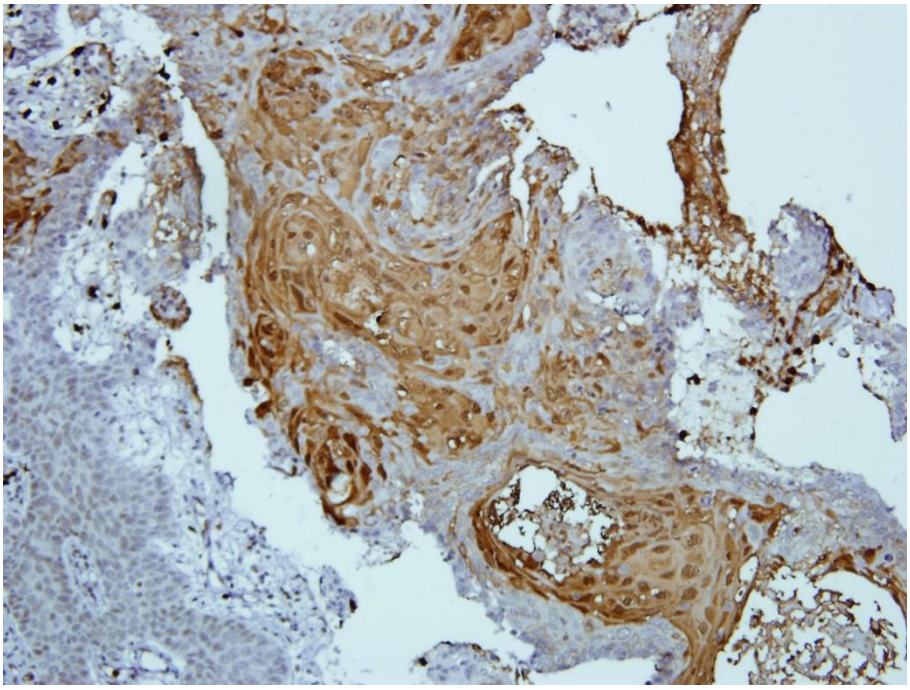

Figure 16: HIF 1α expression in the poorly differentiated squamous cell carcinoma (20x Magnification.)

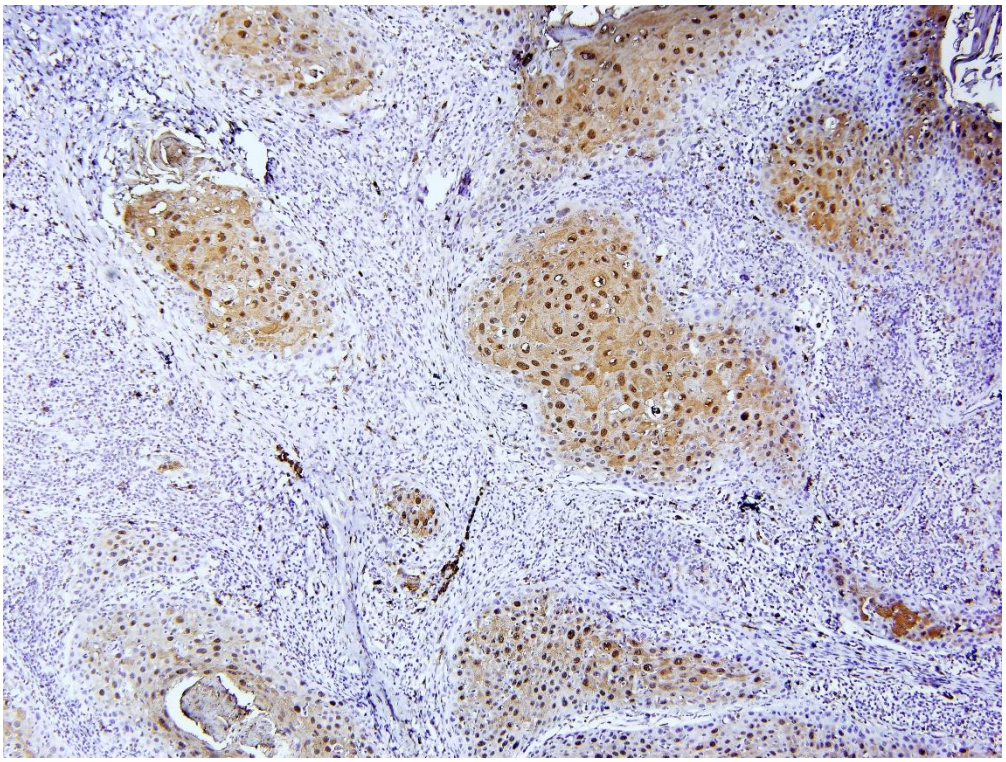

Figure 17: Tumor cell islands expressing the HIF 1α in the poorly differentiated squamous cell carcinoma. . (10x Magnification.)

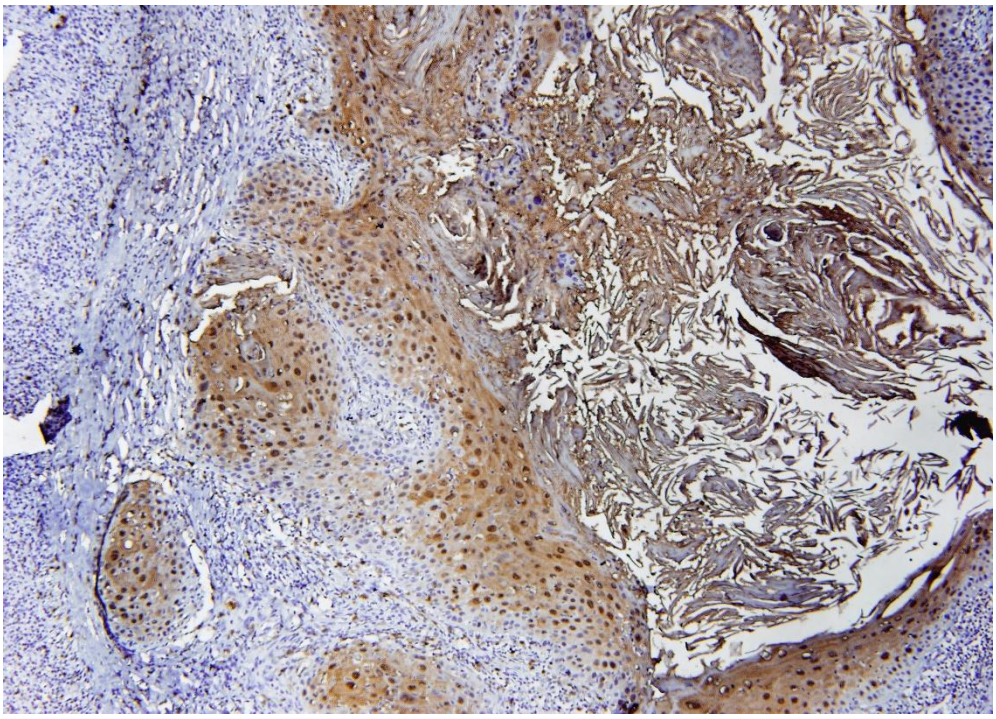

Figure 18: The HIF 1α expressed in the tumor cells nearer to the keratin flecks of the poorly differentiated squamous cell carcinoma.(10x Magnification.)

Discussion

The coral pink color of the healthy oral mucosa is attributed to the rich vascular supply present in the supporting connective tissue visible through the translucent epithelium. An increased epithelial proliferation as a result of any proliferative stimulus leads to a thickened epithelium with concurrent absorption of water from the saliva giving rise to the whitish appearance of the oral mucosa. Leucoplakia's are such an altered epithelial lesions which are whitish unscrappable caused due to the abuse of the tobacco and its products. Leucoplakia may show different grades of altered growth and maturation of the epithelial cells when viewed under microscope and are termed dysplastic epithelia, the grading of which is dependent on the level of the strata involvement.

In the oral cavity, one of the most common potentially malignant lesion is leucoplakia, but it is also apparent that as many as 50% of oral squamous cell carcinomas arise from apparently clinically normal mucosa.[82] Histopathologically oral epithelial dysplasia (OED) is regarded as the standard terminology although some texts use terms such as the Squamous Intraepithelial neoplasia or squamous intraepithelial lesions for epithelial dysplasia but the terminology is not exactly synonymous. The malignant transformation of OED to OSCC (oral squamous cell carcinoma) is evident in the literature but the actual mechanisms are poorly understood. The present study is an attempt to know the hypoxic state of oral epithelial dysplasia and the oral squamous cell carcinoma.

Epithelium as a tissue is devoid of its own blood supply and the sustenance comes through the diffusion of the nutrients through the basement membrane outsourced by the richly vascularized underlying supportive connective tissue. Increased proliferation of the epithelial cells in the dysplastic epithelium leads to the areas of low oxygen partial pressures occurring either acutely or chronically should be aptly facilitated by formation of new blood vessels termed as neo angiogenesis to supply the oxygen and the nutritional needs for the tumor progression and such an angiogenic switch is dependent mainly on hypoxia which is under the regulation of HIF 1α.

Even though quantitative antigen information of HIF1α is possible with the biochemical assays and flow cytometry, the main disadvantage is that there is the loss of an observable relationship between the antigen and tissue morphology. Hence the present study is aimed at immunohistochemical analysis of HIF1α which conserves the spatial relationship between the antigen and tissue morphology. The combined immunohistochemistry and the computer assisted image analysis will help in the objective quantification of the HIF 1α in OED and OSCC.As HIF 1α antigen expression is heterogeneous in OED and OSCC sections, a combination of immunohistochemistry and computer assisted image analysis was needed to know the antigen distribution and the objective assessment of the quantification of primary antibody HIF 1α in OED and OSCC.

The dysplastic cell and the cancer cell tries to adapt to the hypoxia/hypoxemia/anoxia by the transcriptional induction of a series of genes that participate in the neo angiogenesis, iron metabolism, glucose metabolism and cell proliferation and cell survival. The primary factor mediating this response is the HIF 1α, an oxygen sensitive transcriptional factor with short half-life ($t_{1/2}$~5min) and is highly regulated by oxygen.[1]

Daniel M.Aebersold et al mentions that in all the HIF 1α positive tumor samples immunostaining was nuclear; in rare instances weak cytoplasmic reactivity was also observed in oropharyngeal cancer which is similar to the oral squamous cell carcinoma in the present study. The two predominant patterns of nuclear staining encountered by them as focal expression, with intense reaction occurring distal to the closest blood vessel and surrounding necrotic areas and diffuse expression, independent of the vessel proximity similar to the present study depicted as in the Fig.17 & 18. In the Oropharyngeal carcinomas of their study, focal and diffuse expression constituted 65% and 35% respectively but in the present study OSCC focal and diffuse expression is 68% and 32% respectively, with a minimal variation of HIF 1α pattern expression.[12]

In the present study, poorly differentiated OSCC showed the expressivity of the HIF 1α more than in well differentiated OSCC. This could be due to the clonal variation of the primary antibody.

Heba El-Sayed Mohammed Youssef et al study the well differentiated OSCC showed cytoplasmic HIF 1α was predominant than nuclear staining accounting for 63% of cases while in the present study it accounted for 70%.Moderately differentiated OSCC in their study showed nuclear HIF 1α predominant than the cytoplasmic for 82.3% but the present study showed 80%.Poorly differentiated cases stained accounted

for 77% but the present study data indicates it to be 70%.Heba El-Sayed Mohammed Youssef et al results revealed the whole nuclear HIF1α staining of OSCC was around 45% and Gui-quan Zhu et al states the nuclear HIF1α positivity to be 52.5% but the present study positivity is 73.3%.Such a variation in staining intensity might be due to patients gender, age, T stage, lymph node involvement, histologic differentiation, neck dissection, post-operative radiotherapy or post-operative chemotherapy. The hypoxia state increases as the distance of the sample site from the blood vessel increases leading to the conflicting results. E.Brouwer et al studied HIF1α in angiogenesis and inflammation in rheumatoid arthritis with four different monoclonal antibodies where some showed nuclear staining and some the cytoplasmic similarly the variability in the percentage of staining of HIF1α in the above studies as compared to the present study might be due to the usage of the different clonal antibody.[41]

Histologically, the localized HIF 1α expression around keratinized and necrotic areas of the tumor as depicted in the well differentiated OSCC case (fig13) or in the regions distal to the nearest blood vessel appeared to corroborate with the observations of the previous studies.[49,92,87] This supports the hypothesis that HIF-1α expression is induced mainly by hypoxia and the presence of blood vessels in the tumor stroma does not necessarily ensure that a region of tumor is receiving appropriate perfusion and oxygenation. This could be due to the tumor blood flow that may be transiently compromised in regions where micro vessels are immature, deformed or occluded. Moreover, areas where cells beyond the diffusion distance of oxygen from micro vessel may be subjected to chronic hypoxia that strongly induce cells to produce HIF-1α as an adaptive mechanism to this hypoxia.[91, 92] In the present study Immunohistochemical staining confirmed expression of HIF 1α as clear and brown particles as well as dark and diffuse brown staining located in the cytosol and nuclei of the dysplastic and the tumour cells. With carcinogenesis, the rate of expression of HIF 1α increased and there was significant higher intensity of its expression in the dysplastic and tumour cells than in the normal oral mucosa. The extent, intensity and distribution of HIF 1α staining seen is heterogeneous and regularly found around the necrotic areas of the oral squamous cell carcinoma.

Various study methods available for semi quantitative analysis of Immunohistochemical markers are ARS (Allred Score method where Allred Score=Proportion score+Intensity score), Quick score system(Both multiplication and addition of % of positive cells and Intensity of staining), HSCORE system (Histology Score),Immuno Reactive Score (IRS = % of positive cells x Intensity of staining) by Remmele and Stegner method and Average Threshold Method(ATM).[102,103,104.]Area based method such as ATM is a more accurate approach for a typical IHC image of both the normal and the neoplastic tissue present with positive tissue variable in both extent and intensity. Kingshuk Roy Choudhury et al states that the ATM score and manual scoring by an expert pathologist showed that both methods result in identical scores.[102] Besides the availability of various methods of the semi quantitative analysis, here is a manual method for semi quantitative analysis of the HIF 1α in OED and OSCC which is suitable to compare and most widely followed.

Semi quantitative analysis of HIF 1α was assessed by adding the points of staining intensity and percentage of positive cells for the primary antibody HIF 1α similar to the methodology of the Peter Birner et al in the early invasive cervical cancer and epithelial ovarian tumours, and Barbara Bachtiary et al in radical radiotherapy for cervical cancer.[14,15,23] Here is a table of comparison of the present study values in percentage of expression of HIF 1α in OED and OSCC with the above studies who followed a similar methodology of assessing the staining of HIF 1α as stated in the annexure 3.

The reasons for variable expressivity OF HIF 1 may be due to

- p53 upregulated during hypoxia negatively influences HIF 1α stimulated transcription.[14]
- HIF 1α expression differed depending on the inactivation potential of the HPV type found in the tissue.[23]
- V-SRC(viral sarcoma, a proto oncogene),growth factors like Insulin growth factor, fibroblast growth factor and epidermal growth factor and activation of pathways like phosphatidyl inositol 3 kinase, AKT pathway, FRAP(FKBP-Rapamycin Associated Protein) pathways, all of which increase HIF 1α expression.[12,28]

Mean score of the expression of HIF 1α in nucleus, cytoplasm and nucleus as well as cytoplasm accounted for 63.47, 87.68 and 72.98 respectively which states that under hypoxia, HIF 1α cytoplasmic expression alone is the greatest than the nuclear expression and nuclear and cytoplasmic expression. In such areas of low pO_2, the prolyl hydroxylases are inhibited leading to an accumulation of HIF1α in the cell cytoplasm. The nuclear expression is due to the dimerization of the HIF1α with the HIF1β to form HIF1 which is responsible for the activation of the several target genes.

Additionally, the nuclear and cytoplasmic expression of HIF-1α was more predominant and intense in advanced stages of the epithelial dysplasia and increased degree of malignancy of OSCC in the study, with p value insignificant in the intense group (**p=0.469**) while significant in the mild and moderate stained groups(**p=0.015 & p=0.01** respectively).The shuttling nature of the HIF1α between cytoplasm and the nucleus showed insignificant p value in the nucleus and the cytoplasm(**p=0.496**) and significant in the nucleus **(p=0.005)** and in the cytoplasm(**p=0.038**). These observations were in accordance with previous studies made by Ogane et al. [86] and Zhu et al. [84], who evaluated HIF-1α expression in Squamous cell carcinoma focusing on the nuclear pattern in comparison to the cytoplasmic expression. They found that nuclear pattern predominance is considered to be strongly related to the activated status of HIF-1α as it becomes trans-located to the nucleus to dimerize with HIF-1β subunit to form HIF-1 molecule that may control many pathways involved in aggressive behaviour of the tumors.[87, 49]

The diffuse staining pattern noted in the present study can be explained as not hypoxia related but due to the alterations in the oncogene or tumour suppressor genes but this pattern staining is non-functional in breast carcinomas. Areas of keratinization in the OED and keratin pearls in the OSCC show positivity for the HIF 1α diffuse stating that the HIF 1α has physiological role in the differentiation. Hence HIF 1α can be an epiphenomenon than a carcinogenetic event in the OED and OSCC.[99]

The focal staining pattern was assessed in the study among the 7 cases of WDOSCC, HIF 1α expression was predominantly evident at the periphery of the tumour islands and absent in the central keratin pearls i.e. a prostromal pattern and in the 7 cases of PDOSCC, HIF 1α expression was anti stromal pattern with higher expression in the centre and perinecrotic zones. Similar kind of staining was seen in a study on GLUT1 immunoexpression in oral epithelial dysplasia, oral squamous cell carcinomas and verrucous carcinomas by Vidya C.Angadi in 2015 which indicates that GLUT1 is regulated by HIF 1 α in OED and OSCC.[79]Thus the HIF 1α induction following hypoxia involves a succession of changes to its intrinsic activity, kinetics and expression in OED and OSCC.

In the present study 1 mild dysplasia, 3 moderate dysplasia, 1 severe dysplasia, 3 well differentiated OSCC, 2 moderately differentiated OSCC and 3 poorly differentiated OSCC cases revealed no positivity for the HIF 1α and there is no certain explanation for this finding in the study. However prolonged fixation of the OED and OSCC cases is known to substantially compromise the antigen detection so that failure to stain these cases might be artificial. The other reason for the negativity of the HIF1α expression is that the HIF 1α might be at levels below the limits of detection by IHC technique.[49]

Among the 30 OED stained cases, 25 cases (83.3%) expressed HIF 1α with varying staining densities and 5 OED cases unstained. The stained 16 OED cases showed no significant positivity except for very mild positivity in few areas. It was noted that in some cases with epithelial dysplasia showed HIF 1α positivity that was mostly restricted to dysplastic cell zone as depicted in severe dysplasia case depicted in fig.9. The two severe epithelial dysplasia cases depicted in the Fig. (5, 6, 7, and 8) reveal that within the severe epithelial grading one shows nuclear expression and the other shows the cytoplasmic expression stating that within the same grading dysplastic case shuttling nature of the HIF 1 α from nucleus to cytoplasm is variable. While the mild epithelial cases depicted in Fig. (3,4,5,6) states that the HIF 1α localization in the various layers of the epithelium is varied like one expressing in the basal cell layer, the other in the superficial one third of the stratum spinosum and the another in the entire stratum spinosum. Such a kind of variable expressivity in the different strata's of the OED has yet to be answered and what are the initiating factors for this are to be known.

More over the relevance of the present study cannot be overstressed specially in the context of the Indian subcontinent, where the prevalence of leucoplakia and carcinomas is one of the highest in the world, attributed to the wide spread abuse of tobacco and its products by the general public. Also, scientific literature is abound with information regarding proliferative potential of epithelium, evasion of apoptosis,

genetic mutation and other molecular events occurring in hypoxic environment of the dysplasia and carcinomas, an evaluation of the logistics support provided by the HIF 1 α in regulating the hypoxic environment of the cells sustaining in dysplasia and malignancy.

Summary and conclusion: The present study was designed to analyse the distribution, intensity and localisation of HIF 1α expression in OED and OSCC. The results were tabulated and statistically analysed. The demographic and clinical details observed in this study were in accordance with previous literature.25 cases of OED and 22 cases of OSCC showed positivity for HIF 1α accounting for about 83.3% & 73.3% staining respectively. Focal staining was more predominant than the diffuse in both the OED and OSCC with the Focal: Diffuse ratio of around 16:9 &15:7 respectively. Localisation of staining varied from nucleus, cytoplasm and both nucleus and cytoplasm. Predominance of cytoplasmic staining was observed in OED & OSCC than the cytoplasmic staining. A prostromal pattern of staining was seen in WDSCC and anti-stromal pattern of staining was noted in the PDSCC.Intensity of staining was variable from mild to intense and focal clusters of tumour cells in both the lesions showed intense staining reaction irrespective of intensity of the adjacent cells.

1. There is a predominant staining in the basal cells of Mild Dysplasia cases while few cases showed faint expression in the stratum spinosum.
2. The HIF 1α staining was absent in the basal and para basal cell layers of the severe dysplasia cases.
3. Overall score (by addition method) evaluated by scoring system in OSCC showed highest score of 12 in moderately stained group followed by score of 10 in weakly stained group and 7 score in the no staining group. While in OED cases scores of 5,13,5 & 7were obtained for No staining, weak staining, moderate staining and Strong Staining respectively.
4. The study showed a significant elevation in both the nuclear and cytoplasmic HIF1 α expression from NOM through mild OED to moderate or severe OED, suggesting that the expression of HIF 1α is an early event in oral carcinogenesis and the results indicate that the HIF 1 α expression can be a biomarker for the prediction of the progression of OSCCs.

HIF 1α is a known marker for hypoxia in tissues and have a role in cell growth, proliferation and survival of the OED and OSCC. Increased expression of HIF1α is associated with increased hypoxic condition and correlates with the aggressive behaviour of the dysplasia and tumours. However, HIF 1α alone may not predict the aggressiveness of a lesion but instead it should be correlated with other basic markers or histological grading. The full biological significance of HIF 1α in OED &OSCC has to be explored. Further studies with more number of samples and more sophisticated techniques such as cell culture studies and molecular analysis, are needed to ascertain the role of HIF1α in the pathogenesis of the OED and OSCC. The present study demonstrated that HIF 1α has a consistent role in the OED cases and malignant cases and that its expression level and activity appear to be associated with malignant transformation and aggressiveness. The increased HIF 1 α expression associated with the degree of dysplasia reflects the expanding hypoxia. Thus HIF 1α may be a biomarker of certain types of human malignancies and may play an important role in human carcinogenesis.

References

1. Gustav E Lienhard, Jan W Slot, David E James, Mike M Mueckler. How cells absorb glucose. Scientific American January 1992.
2. Robert Augustin. The protein family of glucose transporter facilitators: It's not onlyabout glucose after all. IUBMB life 2010;62(5):315-33.
3. Anthony Carruthers, Julie Dezutter, Amit Ganguly and Sherin U. Devaskar. Will the original glucose transporter isoform please stand up! Am J Physiol Endocrinol Metab. 2009;297(4):e836–e848.
4. J Philips Sapp, Lewis R Eversole and George P Wysoki. Odontogenic tumors. Contemporary oral and maxillofacial pathology 2nd edition. Mosby publishers; 2003. p.134-163.
5. Regezi, Sciubba and Jordan. Odontogenic tumors. Oral pathology clinical pathologic correlations 4th edition. Missouri: Sanduers publishers; 2003. p.267.

6. Rebeca Souza Azevedo, Márcia Grillo Cabral, Teresa Cristina Ribeiro Bartholomeu dos Santos, Albanita Viana de Oliveira, Oslei Paes de Almeida, and Fábio Ramôa Pires. Histopathological Features of Keratocystic Odontogenic Tumor: A Descriptive Study of 177 Cases From a Brazilian Population. International Journal of Surgical Pathology 2012;20(2):154–60.
7. Jing W, Xuan M, Lin Y, et al. Odontogenic tumours: a retrospective study of 1642 cases in Chinese population. Int J Oral Maxillofac Surg. 2007;36:20-25.
8. Hai- Yan Lou, Tie- Jun Li. Odontogenic tumors: A study of 1309 cases in Chinese population. Oral oncology 2009;45:706-711.
9. Richard Ck Jordan, Paul M Speight. Current concepts of odontogenic tumours. Diagnostic histopathology 2009;15(6):303-310.
10. Stavroula J. Theodoroua, Daphne J. Theodorou, David J. Sartoris. Imaging characteristics of neoplasms and other lesions of the jawbones. Part 1. Odontogenic tumors and tumorlike lesions. Clinical Imaging 2007;31:114–119.
11. Peter A. Reichart, Hans P. Philipsen. Classification of odontogenic tumors and allied lesions. In: Riechart P. (Peter), editor. Odontogenic tumors and allied lesions, London: Quintessence publishers; 2004. p. 21-23.
12. Suk Keun Lee, Yeon Sook Kim. Current concepts and occurrence of epithelial odontogenic tumors: I. Ameloblastoma and adenomatoid odontogenic tumor. The Korean journal of pathology 2013;47:191-202.
13. Peter A. Reichart, Hans P. Philipsen. Benign neoplasms and tumor-like lesions arising from the odontogenic apparatus showing odontogenic epithelium with mature fibrous stroma, without ectomesenchyme. In: Riechart P. (Peter), editor. Odontogenic tumors and allied lesions, ed. London: Quintessence publishers; 2004. p. 41-42.
14. Peter A. Reichart, Hans P. Philipsen. Solid/multicystic ameloblastoma. In: Riechart P. (Peter), editor. Odontogenic tumors and allied lesions, ed. London: Quintessence publishers; 2004. p. 43-58.
15. Peter A. Reichart, Hans P. Philipsen. Unicystic ameloblastoma. In: Riechart P. (Peter), editor. Odontogenic tumors and allied lesions, ed. London: Quintessence publishers; 2004. p. 77-86.
16. Khaled Shaikhi, Mirdza Neiders, Frank Chen, Alfredo Aguirre. Morphological variants of ameloblastoma and their mimickers. North American journal of medicine and science 2012;5(1):20-28.
17. Beena VT, Choudhary K, Heera R, Rajeev R, Sivakumar R, Vidhyadharan K. Peripheral ameloblastoma: a case report and review of literature. Case Rep Dent 2012; 2012: 571509.
18. Sivapathasundaram B, Einstein A, Syed Rafiuddeen I. Desmoplastic ameloblastoma in Indians: report of 5 cases and review of literature. Indian J Dent Res 2007;18(4):218-221.
19. Shear M, Speight PM. Odontogenic keratocyst. In: Shear M, editor. Cysts of the oral and maxillofacial regions, 4th edition. Oxford: Blackwell Munksgaard, 2007. p. 6-58.
20. Michael W. Finkelstein, John W. Hellstein, Kimberly S. Lake, Steven D. Vincent. Keratocystic odontogenic tumor: A retrospective analysis of genetic, immunohistochemical and therapeutic features. Proposal of a multicentric study. OOOO 2013;116(1):75-83.
21. M. Vered, O. Peleg, S. Taicher, A. Buchner. The immunoprofile of odontogenic keratocyst (keratocystic odontogenic tumor) that includes expression of PTCH, SMO, GLI-1 and bcl-2 is similar to ameloblastoma but different from odontogenic cysts. J oral pathol med 2009;38:597-604.
22. Michele Regina Nadalin, Eduardo Rodrigues Fregnani, Yara Teresinha Correa Silva-Sousa, Danyel Elias da Cruz Perez. Syndecan-1 (CD138) and Ki-67 Expression in Odontogenic Cystic Lesions. Braz Dent J 2011;22(3):223-229.
23. Merva Soluk Tekkesin, Sevcihan Mutlu, Vakur Olgae. The role of RANk/RANKL/OPG signaling pathways in osteoclastogenesis in odontogenic keratocysts, radicular cysts and ameloblastomas. Head and neck pathol 2011;5:248-253.
24. Merva Solu k TekkeşİN, Sevcihan Mutlu, Vakur Olgac. Expressions of bax, bcl-2 and Ki-67 in Odontogenic Keratocysts (Keratocystic Odontogenic Tumor) in Comparison with Ameloblastomas and Radicular Cysts. Turkish journal of pathology 2012;28(1):49-55.
25. Shokoofeh Jamshidi, Massoumeh Zargaran, Fahime Baghaei, Setareh Shojaei, Reza Zare Mahmoodabadi, Arash Dehghan, Abbas Moghimbeigi. An Immunohistochemical Survey to Evaluate

the Expression of CD105 and CD34 in Ameloblastoma and Odontogenic Keratocyst J Dent Shiraz Univ Med Sci 2014;15(4):192-198.

26. Yogesh Lakkasetty, Narayan Venkatraman, Balasundarishreedhar, Shashidara, Leekymohanty, Shivakumar Madappa Shivamalappa. The expression of cathepsin-D in odontogenic cysts and tumors: Immunohistochemistry study. Journal of Advanced Clinical & Research Insights 2015;2:67–71.
27. Haike Ghazarian, Brian Idoni, and Steven B. Oppenheimer. A glycobiology review: carbohydrates, lectins, and implications in cancer therapeutics. Acta Histochem. 2011;113(3):236–247.
28. Robert K Murray, Daryl K Grany, Peter A Mayer and Victor W Rodwell. Harper's Biochemistry: Carbohydrates, 25th edition. USA: Lange medical books; 2000.
29. Andrea Scheepers, Hans Georg Joost, Annette Schurmann. The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. Journal of parenteral and enteral nutrition 2004;28(6):364-71.
30. Feng-Qi Zhao and Aileen F. Keating. Functional Properties and Genomics of Glucose Transporters. Current Genomics 2007;8:113-128.
31. Matthias A. Hediger, Michael F. Romero, Ji-Bin Peng, Andreas Rolfs, Hitomi Takanaga, Elspeth A. Bruford. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Pflugers Arch - Eur J Physiol 2004;447:465–468.
32. Nobert Avril. GLUT1 expression in tissue and ^{18}F-FDG uptake. The journal of nuclear medicine. 2004;45(6):930-932.
33. Dong Deng, Chao Xu, Pengcheng Sun, Jianping Wu, Chuangye Yan, Mingxu Hu, Nieng Yan. Crystal structure of the human glucose transporter GLUT1. Nature 2014.
34. Andrei R. Manolescu, Kate Witkowska, Adam Kinnaird, Tara Cessford, and Chrisheeseman. Facilitated Hexose Transporters: New Perspectives on Form and Function. Physiology 2007;22:234–240.
35. Xi Li, He Sheng Luo, Shelley C Paul, Tao Tang, Guang Jin Yuan. Down regulation of the expression of GLUT1 plays a role in apoptosis induced by sodium butyrate in HT-29 cell line. International journal of molecular sciences 2006; 7:59-70.
36. Mamoun Younes, Lia V. Lechago, Jacqueline R. Somoano *et al.* Wide Expression of the Human Erythrocyte Glucose Transporter GLUT1 in human carcinomas. Cancer Research 1996;56:1164-67.
37. Wan Seop Kim, Young Youl Kim, Se Jin Jang, Kunchan Kimm, Myeong Ho Jung. Glucose transporter 1 (GLUT1) expression is associated with intestinal type of gastric carcinoma. J Korean med sci 2000;15:420-4.
38. Satoshi Ito, Toshio Fukustao, Takahiro Nemoto, Hisahiko Sekihara, Yousuke Seyama, Shunichiro kubuta. Coexpression of glucose transporter 1 and matrix metalloproteinase-2 in human cancers. Journal of national cancer institute 2002;94(14):1080-91.
39. Martin Kunkel, Torsten E. Reichert, Peter Benz, Hans Anton Lehr *et al.* Over expression of GLUT1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma. American cancer society 2003;97:4.
40. Martin Kunkel, Maximilian Moergel, Marcus Stockinger, Jong Hyeon Jeong, Gerhardt Fritz, Hans Anton Lehr, Theresa L Whiteside. Overexpression of GLUT1 is associated with resistance to radiotherapy and adverse prognosis in squamous cell carcinoma of the oral cavity. Oral oncology 2007;43:796-803.
41. Ayhan Ozcan, Steven S. Shen, Qihui Jim Zhai, Luan D. Truong. Expression of GLUT1 in primary renal tumors. Morphologic and biological implications. Am J clin pathol 2007;128:245-54.
42. Fu-Yen Chung, Ming-Yii Huang, Ching-Sheng Yeh, Hui- Jen Chang, Tian-Lu Cheng, Li-Chen Yen, Jaw-Yuan Wang and Shiu-Ru Lin. GLUT1 gene is a potential hypoxic marker in colorectal cancer patients BMC Cancer 2009;9:241
43. Ka tia C. Carvalho, Isabela W. Cunha, Rafael M. Rocha *et al.* GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker. Clinics 2011;66:965-972.
44. Jyotsna M Harshani, Sivaranjani Yeluri, Venkateswara Rao Guttikonda. Glut-1 as a prognostic biomarker in oral squamous cell Carcinoma. JOMFP 2014;18(3):372-378.
45. Denise Helen Imaculada Pereira de Oliveira, Ericka Janine Dantas da Silveira, Ana Miryam Costa de Medeiros, Pollianna Muniz Alves, Lelia Maria Guedes Queiroz. Study of the etiopathogenesis and

differential diagnosis of oral vascular lesions by immunoexpression of GLUT-1 and HIF-1α. J Oral Pathol Med 2014;43:76–80.
46. ACBR Johann, JT Salla, RS Gomez, MCF de Aguiar, B Gontijo, RA Mesquita. GLUT1 in oral benign vascular lesions. Oral diseases 2007;13:51-55.
47. Hiroko Ida-Yonemochi, Mitsushiro Nakatomi, Hidemitsu Harada *et al.* Glucose uptake mediated by glucose transporter 1 is essential for early tooth morphogenesis and size determination of murine molars. Developmental biology 2012;363:52-61.
48. Mitsunobu Otsuru, Takayuki Aoki, Keiichi Tsukinoki, Yoshihide Ota *et al*. Usefulness of 18F-Fluorodeoxyglucose Positron Emission Tomography for detecting ameloblastoma, with special reference to glucose transporter 1 expression. J Oral Maxillofac Surg 2008;66:1761-65.
49. Vidya C. Angadi and Punnya V Angadi. Glut1 immunoexpression in oral epithelial dysplasia, oral squamous cell carcinoma, and verrucous carcinoma. Journal of oral science 2015;57(2):115-22.
50. Paola Parente, Antonella Coli, Guido Massi, Antonella Mangoni, Manuela M Fabrizi and Giulio Bigotti. Immunohistochemical expression of the glucose transporters Glut-1 and Glut-3 in human malignant melanomas and benign melanocytic lesions. Journal of Experimental & Clinical Cancer Research 2008;27:34.
51. Reichart PA, Philipsen HP, Sonner S. Ameloblastoma: biological profile of 3677 cases. Eur J Cancer B Oral Oncol. 1995;31B(2):86-99.
52. Sung Soo Kang *et al*. Clinical significance of glucose transporter (GLUT1) expression in human breast carcinoma. Jpn J cancer res 2002;93:1123-8.
53. Zamora-Leon, S. Golde, D. Concha, Delgado-Lopez F, Baselga J, Nualart F and Vera J. expression of the fructose transporter GLUT5 in human breast cancer. Proc Natl Acad Sci 1996;93:1847-52.
54. Cell proliferation and apoptosis in ameloblastomas and keratocystic odontogenic tumors Braz dent j 2012;23(2):91-96.
55. Mervyn Shear. The aggressive nature of the odontogenic keratocyst: is it a benign cystic neoplasm? Part 2. Proliferation and genetic studies. Oral Oncology 2002;38:323–331.
56. Seyed Mohammad Razavi, Hamid Poursadeghi, Atousa Aminzadeh. Immunohistochemical comparison of cyclin D1 and P 16 in odontogenic keratocyst and unicystic ameloblastoma. Dental research journal 2013;10(2):180-3).
57. Glaucia Cardosa Paixao Mateus *et al.* cell proliferation and apoptosis in keratocystic odontogenic tumors. Med oral patol oral cir bucal 2008;13(11):E697-702.
58. Vuhahula E, Nikia H, Ijuhin N, Ogawa I, Takata T, Koseki T, Tanimoto K. Jaw cysts with orthokeratinisation: analysis of 12 cases. J oral pathol med 1993;22:35-40.
59. Thayalan Dineshkumar, Nataraj Priyadharsini, U Punitha Gnanaselvi, Srinivasan Sathishkumar, R P Srikanth, A E Nagarathinam. Evaluation and Comparison of Vascular Endothelial Growth Factor Expression between Ameloblastoma and Keratocystic Odontogenic Tumor. Journal of International Oral Health 2015;7(1):48-52.
60. Timothy H. Witney, Laurence Carroll, Israt S. Alam, Anil Chandrashekran, Quang-D_e Nguyen, Roberta Sala, Robert Harris, Ralph J. DeBerardinis, Roshan Agarwal, and Eric O. Aboagye. A Novel Radiotracer to Image Glycogen Metabolism in Tumors by Positron Emission TomographyCancer Res 2014;74(5):1319-28.
61. L Summers. A histochemical investigation into the presence and role of glycogen in human odontogenic cysts and periapical granulomas. Archs oral boil 1973;18:577-82.
62. Xiao Dong Xu, Shi Xiu Shao, Hai Ping Jiang, Yan Wei Cao, Yong Hua Wang, Xue Cheng Yang, You Lin Wang, Xin Sheng Wang, Hai Tao Niu. Warburg effect or reverse Warburg effect? A review of cancer metabolism. Oncol res treat 2015;38:117-22.
63. Weihua Wu and Shimin Zhao. Metabolic changes in cancer: beyond the Warburg effect. Acta Biochim Biophys Sin 2013;45(1)18-26.
64. Peter Vaupel. The Role of Hypoxia-Induced Factors in Tumor Progression. The Oncologist 2004;9:10-17.
65. [1] Safora Seifi ,Ensieh Shafi gh, Ayoub Allaie. Quantitative and qualitative analysis of argyrophilic nuclear organizer regions in follicular cyst, keratocystic odontogenic tumor and ameloblastoma. Journal of Cancer Research and Therapeutics 2011;7(3):280-5.

66. Alireza Behrooz and Faramarz Ismail-Beigi. Stimulation of Glucose Transport by Hypoxia: Signals and Mechanisms. News Physiol. Sci. 1999;14:105-110.
67. Daniel Kaemmerer, Luisa Peter, Amelie Lupp, Stefan Schulz, Jörg Sänger, Richard Paul Baum, Vikas Prasad, Merten Hommann. Comparing of IRS and Her2 as immunohistochemical scoring schemes in gastroenteropancreatic neuroendocrine tumors Int J Clin Exp Pathol 2012;5(3):187-194.
68. Shinichi Ohba, Hiroaki Fujii, Shin Ito, Mitsuhisa Fujimaki, Fumihiko Matsumoto, Masayuki Furukawa, Junkichi Yokoyama, Takeshi Kusunoki, Katsuhisa Ikeda, Okio Hino. Overexpression of GLUT-1 in the invasion front is associated with depth of oral squamous cell carcinoma and prognosis. J Oral Pathol Med (2010) 39: 74–78.
69. Hilary A. Coller. Is Cancer a Metabolic Disease? The American Journal of Pathology 2014;184(1):4-13.
70. Hideki Makinoshima *et al.* signaling through the phosphatidylinositol 3-kinase (PI3K)/ mammalian target of rapamycin (mTOR) axis is responsible for aerobic glycolysis mediated by glucose transporter in epidermal growthfactor receptor (EGFR) mutated lung adenocarcinoma. The journal of biological chemistry 2015; 290(28):17495-504.
71. Patrick S Ward and Craig B Thompson. Signaling in control of cell growth and metabolism. Cold Spring Harb Perspect Biol 2012; 4:a006783.
72. Risa Chaisuparat, Somchai Yodsanga, Silvia Montaner, Burno C Jham. Activation of Akt/mTOR pathway in dentigerous cysts, odontogenic keratocysts and ameloblastomas. OOOO 2013; 116(3):336-42.
73. Xi Li, He Sheng Luo, Shelley C Paul, Tao Tang, Guang Jin Yuan. Down regulation of the expression of GLUT1 plays a role in apoptosis induced by sodium butyrate in HT-29 cell line. International journal of molecular sciences 2006; 7:59-70.
74. Carlotta Granchi, Daniele Fancelli, Filippo Minutolo. An update on therapeutic opportunities offered by cancer glycolytic metabolism. Bioorganic & Medicinal Chemistry Letters 2014; 24:4915–4925.
75. Yanrong Qian, Xuan Wang, Xiaozhuo Chen. Inhibitors of glucose transport and glycolysis as novel anticancer therapeutics. World J Transl Med 2014; 3(2):37-57.