STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS

Dissertation submitted to

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY

In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

APRIL 2016

CERTIFICATE

This is to certify that this dissertation titled **"STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS"** is a bonafide dissertation performed done by **I. JOSEPH** under our guidance during his postgraduate study period between 2013-2016.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

RAGAS DENTAL COLLEGE Chennai

Dr. K. Ranganathan, MDS, MS(Ohio), PhD
Professor & HOD
Department of Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr. Elizabeth Joshua, MDS
Professor
Department of Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D,
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr. Elizabeth Joshua, MDS
Professor
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr. S. Ramachandran, MDS
Principal
Ragas Dental College & Hospital, Chennai

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS"** is a bonafide and genuine research work carried out by me under the guidance of **Prof. Dr. K. Ranganathan, M.D.S., MS (Ohio)., PhD.,** Professor and Head and **Prof. Dr. Elizabeth Joshua, M.D.S** Professor, Department of Oral & Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

Date: 11.01.16

Place: Chennai

Dr. I. Joseph
Post Graduate Student
Department of Oral & Maxillofacial Pathology,
Ragas Dental College and Hospital,
Chennai.

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI

DECLARATION BY THE GUIDE

I hereby declare that this dissertation titled **"STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS"** is a bonafide and genuine research work carried out by **Dr. I. Joseph**, Post Graduate Student in the Department of Oral & Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai under my guidance in partial fulfillment for the requirement of the degree of **Master of Dental Surgery** (Oral Pathology and Microbiology).

Prof.Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD
Professor & HOD
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D,
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Date: 11.01.16
Place: Chennai

Prof. Dr. Elizabeth Joshua, M.D.S.
Professor
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. Elizabeth Joshua, MDS
Professor
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Date: 11.01.16
Place: Chennai

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI

ENDORSEMENT BY THE HEAD OF THE DEPARTMENT AND HEAD OF THE INSTITUTION

This is to certify that this dissertation titled **"STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS"** is a bonafide dissertation performed by **Dr. I. Joseph** under our guidance during the postgraduate period 2013-2016. It has not been submitted (partial or full) for the award of any other degree or diploma.

Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD
Professor & HOD
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai.

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D,
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Date: 11.01.16
Place: Chennai

Dr. S. Ramachandran, M.D.S
Head of the Institution
Ragas Dental College & Hospital
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

Date: 11.01.16
Place: Chennai

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

CHENNAI

COPYRIGHT

DECLARATION BY THE CANDIDATE

I hereby declare that the **The Tamilnadu Dr. M.G.R. Medical University, Tamilnadu** shall have the right to preserve, use and disseminate this research work in print or electronic format for academic / research purpose.

Date: 11.01.16

Place: Chennai

Signature of the Candidate

Dr. I. Joseph

Acknowledgement

ACKNOWLEDGEMENT

".....for thou blessest, O Lord, and it shall be blessed for ever."(1Chronicles 17:27)

Yes. At the outset, I thank my God the Almighty for enabling me to complete this dissertation with His blessing!

I take it as my great privilege to honour, to remember with gratitude my post graduate teacher ***Dr. K. Ranganathan****, MDS, MS (Ohio), PhD, Professor and Head of Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for his valuable support and encouragement in guiding me in this dissertation, without which it may not have become possible for me to complete this study. His corrections and admonitions, I always cherish.*

At the same time never can I forget ***Dr. UmaDevi. K. Rao****, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for her valuable guidance at every stage of this dissertation. Giving me her precious time educating me and helping me to make this dissertation something presentable - I regard it with gratitude.*

To be more specific and particular, ***Dr. Elizabeth Joshua****, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital was my mentor throughout my study. She has always been an unseen source of strength to me throughout my postgraduate program and I thank my God Almighty for providing me with such a positive person like her. Her valuable guidance, advice and encouragement mean a lot to me more than mere words!*

*I also earnestly thank Professor, **Dr. T. Rooban**, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for his encouragement and concern. He had been always a saviour in crisis and a person on whom I can back upon.*

*I am also deeply indebted to my principal **Dr. S. Ramachandran** and Chairman **Mr. Kanakaraj**, Ragas Dental College and Hospital for extending their patience and cooperation using the facilities of our institution, at all times.*

*I extend my sincere thanks to Reader **Dr. N. Lavanya** and **Dr. C. Lavanya,** Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for their encouragement.*

*I also thank my senior lectures **Dr. Sudarshan** and **Dr. Kavitha**, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital for their constant support.*

*I am very grateful to our Geneticist & Research Assistant **Mrs. Kavitha**, for guiding me at every stage of my project. Her contribution to my study, I will never forget.*

*I also thank my Biostatistician **Ms. Aarthi,** for giving me her time and helping me in the completion of my study.*

*Not to be forgotten, I am very grateful to our Lab Technician **Mr. Rajan**, for his help at every stage of my study.*

I also thank our Attender, ***Mrs. Vasanthi****, for her constant help during the course of my study.*

I acknowledge gratefully the help of my batch-mates ***Dr. Angaiyarkanni. E, Dr. Deepasri. M, Dr. Divya. B, Dr. Malarvizhi. K*** *and* ***Dr. Saranya.V.*** *I also thank my seniors* ***Dr. Gnanambigai, Dr. Gowri Shankar*** *and* ***Dr. Selvajothi and juniors Dr. Jayashree, Dr. Revathi, Dr. Roxana mam, Dr. Saranya. M*** *and* ***Dr. Vasanthi*** *for their support and encouragement.*

Last but not the least, I must sincerely thank my dad, ***Mr. Immanuel*** *and my mom****, Mrs. Sironmani*** *and my sister****, Miss. Elizabeth Ann*** *for their constant love, understanding, and support throughout my curriculum.*

"Let all glory belong to Him"

ABSTRACT

Background: HIF – 2 alpha is over-expressed in primary and metastatic human cancers, and the level of expression is correlated with tumor angiogenesis and patient mortality. Molecules upregulated by hypoxia (HIF alpha) play a role in progression of fibrosis in OSMF. To ascertain the role of HIF – 2 alpha as a surrogate biomarker in the malignant transformation of Oral Submucous Fibrosis, a potential malignant disorder/ premalignant condition.

Aim and Objectives: To study and compare the expression of HIF – 2 alpha in oral submucous fibrosis (OSMF), Oral Squamous cell carcinoma (OSCC) with history of areca nut usage, OSCC without areca nut usage and normal mucosa by immunohistochemistry.

Material and Methods: Immunohistochemical detection of HIF – 2 alpha was done using monoclonal antibody of clone ep-190b and Poly Excel HRP/DAB chromogen detection system on 51 samples, which included oral submucous fibrosis (OSMF - 11 cases), oral squamous cell carcinoma with history of areca nut usage (OSCC - 15 cases), oral squamous cell carcinoma without areca nut usage (OSCC - 15 cases) and the expression was compared with that of normal mucosa (10 cases). The positive control used for HIF-2 alpha was pre-eclamptic placenta.

Results: All the cases of normal mucosa, OSMF, OSCC with areca nut habit and OSCC without areca nut habit showed HIF-2α expression. Connective tissue staining alone was seen in 27% of OSMF cases and there was a significant difference in the proportion of cases, which had expression in the suprabasal, basal and suprabasal layers of epithelium between the groups. An intense expression of HIF-2α was seen in 13% cases of OSCC associated with areca nut habit. Mild and moderate expression was seen in most cases of OSMF, OSCC and normal control groups. The pattern of HIF-2α staining in most of the cases of normal mucosa, OSMF and OSCC was cytoplasmic and nuclear. All the cases of OSCC with areca nut habit had their staining either cytoplasmic or nuclear and cytoplasmic. 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 the expression of HIF-2α, which increased as the tumor became poorly differentiated.

Conclusion: Areca nut habit induces hypoxia in OSMF which triggers the expression of HIF-2α in the epithelium. HIF-2α could be a marker for cancer initiation and progression.

Key words: HIF-2α, angiogenesis, hypoxia, oral potentially malignant disorder.

CONTENTS

Introduction

Oral Squamous cell carcinoma (OSCC), one of the more common malignant tumors of the head and neck, comprises of 90-95 percent of all oral malignancies. Inspite of the advancement in chemotherapy, radiotherapy, and targeted therapy in the last three decades, the prognosis of OSCC is poor due to its aggressiveness, local invasion and metastasis, leading to recurrence. Thus, in the field of head and neck cancers, the treatment for OSCC is still a challenge[1].

Oral submucous fibrosis (OSMF) is a chronic, insidious and progressive disease that primarily affects any part of the oral cavity. It is characterized by a juxta-epithelial inflammatory reaction followed by progressive fibrosis of the lamina propria and the underlying submucosal layer, with associated epithelial atrophy[2]. OSMF carries a high risk of transition to oral cancer. In one epidemiologic study in India, the transformation rate of OSMF to OSCC was 7.6% to 12 over a period of 17 years[3].

Cancer induction is a multi-stage, multi-step process that includes multiple cellular and molecular events to transform a normal cell into a malignant neoplastic cell. The general steps that can be identified in carcinogenesis are initiation, promotion and progression. Oral squamous cell carcinoma (OSCC) transformed from OSMF is clinically more invasive and also exhibits higher metastasis and rate of recurrence than OSCC that has not transformed from OSMF[4].

With the progression of the disease process of OSMF, extensive fibrosis of the underlying connective tissue causes reduction in vascularity, resulting in subsequent hypoxia in both fibroblasts and the overlying surface epithelium. Hypoxia causes atrophy and ulceration of the epithelium by inducing apoptosis. In addition, hypoxia-inducible factor-1alpha (HIF-1α) is highly expressed in OSMF, which could further indicate changes in cell proliferation, cell maturation and its metabolic adaptation, increasing the possibility of malignant transformation. The hypoxic stress activates solid tumor to over-express a variety of factors such as HIF and vascular endothelial growth factor (VEGF), which enhances abnormal vascular endothelial cell proliferation and differentiation[4].

Another mammalian bHLH-PAS protein similar to HIF-1α, HIF-2α (Hypoxia inducible factor-2 alpha, also called EPAS1/HRF/HLF/MOP2), is responsible in executing the hypoxia response. They are both closely related structurally, sharing 48% overall amino acid identity. HIF-2α also binds ARNT (aryl hydrocarbon receptor nuclear translocator), which is present in most cells and transactivates hypoxia response elements (HRE)-containing genes which target for erythropoietin and vascular endothelial growth factor (VEGF)[5].

HIF-2α is not as ubiquitously expressed as HIF-1α but is found prominently in vascular endothelial cells during embryonic development. In addition to endothelial cells, HIF-2α mRNA has also been found in liver

hepatocytes, kidney fibroblasts, epithelial cells of intestinal lumen, pancreatic interstitial cells, interstitial cells of heart and myocytes, and lung type II pneumocytes. In contrast to restricted expression patterns *in vivo*, almost all transformed cell lines show the expression of HIF-2α. Further, it has been shown to be expressed in vascular cells, parenchymal cells, and infiltrating macrophages in the tumor microenvironment. These data indicate that HIF-2 alpha might play an important role in a broad range of cells in addition to endothelial cells as well as in tumorigenesis[5].

HIF-1α plays a role in endothelial cell proliferation, migration and vessel sprouting, whereas HIF-2α plays a more significant role in controlling the vascular morphogenesis, integrity and the assembly[6]. Since, there is extensive fibrosis, hyalinisation and vascular alterations in the connective tissue stroma of OSMF leading to a hypoxic situation, this study was undertaken to analyze the pattern of immunohistochemical expression of the Hypoxia Inducible factor (HIF) – 2 alpha in submucous fibrosis of the oral mucosa, oral squamous cell carcinoma with history of areca nut usage, oral squamous cell carcinoma without history of areca nut usage and compare it with normal mucosa.

Aims and Objectives

AIM:

To study whether Hypoxia Inducible Factor (HIF) - 2 alpha expression is upregulated in oral submucous fibrosis and oral squamous cell carcinoma.

OBJECTIVES:

1. To study the expression of HIF - 2 alpha by Immunohistochemistry in archival blocks of:

    a) Oral submucous fibrosis
    b) Oral squamous cell carcinoma with history of areca nut usage*
    c) Oral squamous cell carcinoma without history of areca nut usage**
    d) Normal mucosa

2. To compare the expression of HIF - 2 alpha in OSMF, OSCC and normal oral mucosa by Immunohistochemistry.

** areca nut usage:- use of areca/ betel nut (pan), areca with chewable tobacco (betel quid, commercial products like pan parag, mawa, gutkha) by chewing or pouching or swallowing and areca with non-chewable tobacco (smoking) including consumption of alcohol.*

***without areca nut usage:- both chewable and non-chewable forms of tobacco, history of no habits.*

HYPOTHESIS (NULL)

There is no difference in HIF -2 alpha expression in Oral Submucous fibrosis, Oral Squamous cell carcinoma when compared to normal mucosa.

STUDY SETTING:

The study was retrospectively conducted in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using archival formalin fixed paraffin embedded blocks of OSMF, OSCC and normal mucosa using immunohistochemistry.

STUDY SUBJECTS:

The study material comprised of 51 formalin fixed, paraffin embedded tissue specimens. The samples were divided into 4 groups namely: Group I, Group II, Group III and Group IV.

Group I : 11 samples of OSMF

Group II : 15 samples of OSCC associated with areca nut habit

Group III : 15 samples of OSCC without areca nut habit

Group IV : 10 samples of normal mucosa

INSTITUTIONAL REVIEW BOARD

This study was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai (Annexure I).

Materials and Methods

MATERIALS AND METHODS:

1. Tissue samples of normal oral mucosa (n=10), OSMF (n=11), OSCC without areca nut habit (n=15) and OSCC with areca nut habit (n=15) were taken from the archival blocks.
2. A detailed case history including patient's age, gender, occupation, past medical and dental history, history of drugs and trauma were recorded.
3. Extra oral and intra oral examination was done.
4. Biopsy was performed from the lesional site. Normal oral mucosa was taken when the patients were undergoing minor surgery for extraction of impacted teeth.
5. The tissue biopsied was immediately transferred to 10 % buffered formalin.
6. After adequate fixation, tissues were embedded in paraffin.
7. From the paraffin embedded blocks 5 microns thick, sections were cut and used for routine hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) staining.
8. Tissue sections of placenta were used as positive control for HIF – 2α positivity.
9. HIF-2α RNA is highly expressed in vascular tissues such as lung, heart, placenta, and kidney [7]. The HIF-2α immunoreactivity is mainly located in the nuclei of the syncytiotrophoblast, trophoblastic villous

cells and fetoplacental vascular endothelium in the pre-eclamptic villous placenta which are not regulated by hypoxia in placental villous explants. There may be additional reasons which could be due to the oxygen-sensing mechanism in the pre-eclamptic placenta, which may have higher sensitivity or gain, impaired degradation via the ubiquitin-proteosome pathway, and activation by growth factors [8,9].

HEMATOXYLIN AND EOSIN (H & E) STAINING:

REAGENTS

Harris's hematoxylin

1% acid alcohol

Eosin

Xylene

Alcohol

PROCEDURE

The slides were dewaxed in xylene and hydrated through graded alcohol to water. The sections on the slides were flooded with Harris's hematoxylin for 5 minutes. The slides were washed in running tap water for 5 minutes. The slides were differentiated in 1% acid alcohol for 5 minutes. The slides were washed well in running tap water for 5 minutes. The tissue

sections on the slides were then stained in eosin for 30 seconds. The slides were washed in running tap water for 1 minute. The slides were then dehydrated through alcohol, cleared, mounted and viewed under light microscope (LM).

IHC STAINING (HIF-2 alpha):

Armamentarium:-

Microtome

Autoclave

Hot air oven

Slide warmer

Couplin jars

Measuring jar

Weighing machine

APES coated slides (Amino propyl triethoxysilane)

Slide carrier

Aluminium foil

Micro-pipettes

Toothed forceps

Electronic timer

Beakers

Rectangular steel tray with glass rods

Sterile gauze

Cover-slips

Light microscope

REAGENTS USED

1. APES (3 amino propyl tri ethoxysilane)

2. 1N sodium hydroxide

3. 1N HCl

4. Tris EDTA (Ethylene Diamine Tetra Acetate) buffer (50X)

5. Tris buffered Saline with Tween 20

7. 3% H_2O_2

8. Distilled water

9. Hematoxylin

10. Absolute alcohol (Isopropyl alcohol)

11. Xylene

ANTIBODIES USED

1. Primary antibody – Anti-HIF-2-alpha mouse monoclonal antibody-Clone ep-190b, Abcam (Annexure III)
2. Secondary antibody – Poly Excel-HRP Micro polymer IHC detection system- Vkan Life Care (Annexure IV)

PREPARATION OF PARAFFIN SECTIONS

After the slides were dried, tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slides from the tissue float bath such that two tissue bits come on to each slide with a gap in between. One of the tissue sections towards the frosted end of the slide was labelled negative to which negative serum, the secondary and the chromogen were added and the tissue section away from frosted side is the positive to which the primary antibody, secondary antibody and chromogen were added.

IHC PROCEDURE

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Circles were drawn using a diamond marker around the tissues, so that the antibodies added later on do not spread and are restricted to the circle. The slides were transferred to TRIS EDTA buffer of pH 9 and steamed in pressure cooker for antigen retrieval at 15 lbs pressure for

15 minutes. Slides were then treated with 3% polyexcel hydrogen peroxide for 7 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining then the slides were dipped in TRIS buffered saline with Tween 20 for 6 minutes. The slides were wiped carefully without touching the tissue section. The pre diluted primary antibody, HIF-2 alpha was added to positive tissue on the slide and to the negative tissue tris buffered saline was added. The slides were incubated for one hour and thirty minutes. The slides were then wiped carefully without touching the tissue section to remove excess of antibody and washed with tris buffered saline with tween 20 for 2-3 minutes. The polyexcel target binder reagent is then added and incubated for 12 min. The slides were wiped carefully without touching the tissue section to remove excess and washed with tris buffer with tween 20 for 2-3 minutes. A drop of polyexcel poly horseradish peroxidase was then added on both the sections and the slides were incubated for 12 minutes. Later slides were washed in tris buffered saline for 2-3 minutes. The slides were then wiped carefully without touching the tissue section to remove excess buffer. A drop of DAB was added to the sections and incubated for 3 min. Slides were then washed in distilled water to remove excess chromogen and counter stained with hematoxylin. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX. The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

POSITIVE CONTROL

A case of pre-eclamptic placenta specimen known to express HIF-2 alpha positivity were fixed, processed, embedded, sectioned, stained in same manner and used as positive control. One positive control tissue slide was included for each batch of staining. The tissue section away from the frosted end of the slide was taken as the positive control.

IHC PROCEDURE:

1. APES coated slides with 2 paraffin embedded tissue placed in warming table
2. Placed in xylene twice (5 minutes each)
3. Placed in 100% isopropanol (5 minutes)
4. Placed in 90% isopropanol (5 minutes)
5. Placed in 70% isopropanol (5 minutes)
6. Washed in distilled water (2 minutes each)
7. Keep in TRIS EDTA buffer at pH 9 for antigen retrieval
8. Placed in 3% hydrogen peroxide (7 minutes)
9. Washed with Tris buffer (2-3 minutes)
10. Primary antibody added and incubated (1 hour 30 minutes)
11. Washed in Tris buffer (2-3 minutes)
12. Poly excel target binder reagent added and incubated (12 minutes)
13. Washed in Tris buffer (2-3 minutes)
14. Poly excel HRP added and incubated (12 minutes)

15. Washed slides in Tris buffer (2-3 minutes)
16. DAB added and incubated in an enclosed in hydrated container (3 minutes)
17. Washed in Tris thrice (2-3 minutes)
18. Stained with Harris Hematoxylin (20 seconds)
19. Washed in tap water
20. Placed in 70% alcohol (1 minute)
21. Placed in 100% alcohol (1 minute)
22. Placed in xylene (1 dip)
23. Slides to be mounted using DPX
24. Slides to be observed under the LM and graded

CRITERIA FOR EVALUATION OF STAINING:

Evaluation of H & E sections:

- The H&E stained sections were thoroughly examined.
- Oral squamous cell carcinomas were graded as well differentiated, moderately differentiated and poorly differentiated.

Evaluation for IHC:

- Corresponding sections as examined by H & E were stained by IHC to detect HIF-2α expression.
- To all these sections the HIF-2α antibody and the tris buffer saline was added based on the IHC protocol for the HIF-2α antibody.

- The positive control used for IHC was pre-eclamptic placenta
- Positive cells were counted in the basal and suprabasal layers of normal mucosa, oral submucous fibrosis, oral squamous cell carcinoma with areca nut chewing habit and oral squamous cell carcinoma without areca nut chewing habit.
- Percentage of positive cells were also counted in each case and it was categorised as 0= no expression; 1+ = <20% of cells positive; 2+ = 20% to 50%; 3+ = >50%
- Connective tissue was also examined in all the lesions.
- The Mean Labelling Index (MLI) for all the positive groups were calculated using the formula:

$$\frac{\textbf{Number of positive cells X 100}}{\textbf{Total number of cells}}$$

TISSUE LOCALISATION:

HIF-2 alpha stain is localized to the basal, supra basal layers of the epithelium and the connective tissue.

CELLULAR LOCALIZATION OF STAIN

The stain is localized to cytoplasmic portion of the normal epithelial cells and the aberrant expression of HIF-2α is seen in nuclear region of epithelial cell. The stained slides were screened, examined systematically for HIF-2α expression in nuclear and cytoplasmic portion of the epithelial cell.

INTENSITY OF STAINING

The HIF-2 alpha intensity was analyzed in the basal, supra basal layers of epithelium and connective tissue in the study groups.

Each case was graded as (-) nil or absence of stain, (+) mild, (++) moderate and (+++) intensively stained based on the intensity of staining taken up by the tissue as observed by two blinded observers independently with respect to positive control.

STATISTICAL ANALYSIS

Data were entered and analyzed using SPSS^{TM} software (version 21.0). Pearson's Chi-square test was done to compare intensity of staining between the groups. P value ≤ 0.05 was considered statistically significant. Kappa analysis was done to compare the intensity of HIF-2 alpha staining as observed between two observers.

Review of Literature

ORAL SUBMUCOUS FIBROSIS

Oral submucous fibrosis (OSMF) was first described by Schwartz in 1952 as a fibrosing condition that occurred in five Indian women in the country of Kenya and he termed it "Atrophica idiopathic (tropica) mucosa oris'. Oral submucous fibrosis is present in medical literature since the time of a renowned Indian physician, Sushrutha (2500-3000 BC) who described a condition mimicking OSMF which he referred to as 'Vidari'.

It is a potentially malignant disease predominantly seen in people of Asia. Being a chronic progressive disorder, its clinical presentation depends on the stage of the disease at the time of diagnosis. Most patients present with an intolerance to spicy food, rigidity of palate, tongue and lip progressing on to varying degrees of limitation of mouth opening and tongue movement.

The hallmark of the disease is fibrosis of the submucosa that affects most parts of the oral cavity, pharynx and upper third of the oesophagus. Possible etiological factors discussed to date are areca nut, capsaicin, deficiencies of iron, zinc and essential vitamins. Moreover, a possible autoimmune basis to the disease with demonstration of various autoantibodies and an association with specific HLA antigens has been proposed [10].

The association between copper and OSMF has been related to excess copper found in tissues of other fibrotic disorders – Indian childhood cirrhosis, Wilson's disease and primary biliary cirrhosis [10]. The characteristics of copper

and its nature present in areca nut is yet unknown and the solubility of copper complex in physiologic solutions such as saliva are not fully evaluated, but initial findings have shown that soluble copper is extracted into saliva during the chewing of areca products [11].

The malignant transformation rate of OSMF has been reported to be approximately 7.6% over a 17-year period [12].

ETIOPATHOGENESIS

Recent epidemiological studies show that areca nut is the main aetiological factor for OSMF. A clear dose-dependent association was seen for both duration and frequency of chewing areca nut in the development of OSMF. Commercially available freeze dried products of areca such as pan masala, Gutkha and mawa (areca and lime) have high concentrates of areca nut per chew and likely to cause OSMF more rapidly compared to self-prepared conventional betel quid that contain smaller amounts of areca nut [10].

Other factors include ingestion of chillies, genetic and immunologic processes, nutritional deficiencies. Epidemiological studies and histopathological effects on keratinocytes and fibroblasts support areca nut as one of the most important risk factors for OSMF [13].

Areca nut is made up of both alkaloid and flavonoid components. Four alkaloids in areca nut namely arecoline, arecaidine, guvacine, and guvacoline are identified, of which arecoline is the most potent agent and plays a major

role in the pathogenesis of OSMF causing an abnormal increase in the production of collagen. The flavonoid components, tannins and catechins are found to have some direct influence on collagen metabolism [13].

Numerous biological pathways are involved and the normal regulatory mechanisms are either up regulated or down regulated at different stages of the disease. Among the chemical constituents of areca nut, alkaloids are the most important biologically while tannin may have a synergistic action. These chemicals are likely to interfere with the molecular processes of deposition and/or degradation of collagen. Studies done *in vitro* on human fibroblasts using areca extracts or chemically purified arecoline, favour proliferation of fibroblasts and increase in collagen formation that is also demonstrable histologically in human OSMF tissues. The high copper content of areca nut and the possible role of copper as a mediator of fibrosis is demonstrated by up regulation of lysyl oxidase in OSMF biopsies further areca nut may also enhance the development of the disease by increased levels of cytokines in the lamina propria. Increased, continuous deposition in the extracellular matrix may take place as a result of perturbations of the equilibrium between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMP). Genes related to collagen have been linked to the susceptibility and pathogenesis of OSMF. The individual mechanisms operating at the initial, intermediate and advanced stages of disease need further study in order to propose appropriate therapeutic interventions [10].

Basic mechanisms involved in the pathogenesis of OSMF can be divided into four steps:

1. Chronic inflammation at the site of betel quid placement
2. Increased collagen synthesis
3. Cross-linking of collagen
4. Decreased collagen degradation

Chronic inflammation at the site of betel quid placement:

The areca nut chewing habit of placing the betel quid in the vestibule for varying duration and frequency varies among individuals. The alkaloids and the flavonoids from the quid are absorbed due to the constant contact between the betel quid and the oral mucosa, and undergoes metabolism. These constituents along with their metabolites are a constant source of irritation to the oral mucosa. Mechanical irritation of the oral mucosa also occurs along with this chemical irritation due to coarse fibers present in the betel quid. The microtrauma resulting from this continuous friction of coarse fibers of the areca nut facilitates the diffusion of betel quid alkaloids and flavonoids into the subepithelial connective tissue, resulting in infiltration of inflammatory cells juxtaepithelially. Chronic inflammation sets in at the site due to persistent areca nut chewing and is characterized by the presence of activated T cells, macrophages, etc. Synthesis of various mediators of inflammation like

prostaglandins secreted by the oral keratinocytes in response to areca nut extract has been shown.

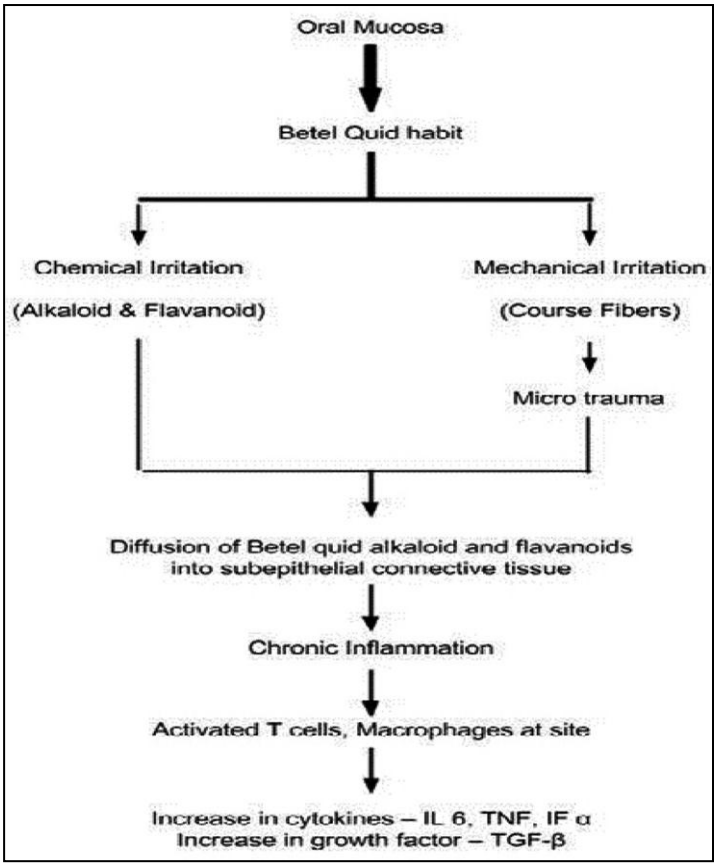


Fig. 1: Schematic representation of chronic inflammatory process at the site of placement of the betel quid)

Cytokines like interleukin 6 (IL 6), tumor necrosis factor (TNF), interferon α and Transforming growth factor – beta (TGF-β) are synthesized at the site of inflammation. Initial irritation leads to further atrophy and ulceration of the mucosa followed by persistent inflammation which is necessary for tissue fibrosis and cancer to occur. So, the induction of

inflammation by betel quid ingredients can be considered, a critical step in the pathogenesis of OSMF [10].

Increased collagen synthesis:

TGF- β is a key regulator of the extra- cellular matrix (ECM) assembly and remodelling. It helps in activation of procollagen genes and increase in pro-collagen proteinase levels (PCP – Procollagen C-proteinase, BMP 1- bone morphogenetic protein1, PNP – Procollagen N-proteinase).

The procollagen genes are transcribed and translated to form pro-collagen monomeric chains (procollagen precursor). Three of these monomers assemble into a trimer triple helix which is aided by disulphide bridge formation. N and C – terminal proteases act on these trimeric procollagen chains to cleave the terminal domains and form the fibrils. The fibrils newly formed are then covalently stabilized through cross-linking to form a stable mature structure of collagen [14].

The genes COL1A2, COL3A1, COL6A1, COL6A3, and COL7A1 have been identified as definite targets for TGF- β. The transcriptional activation of type I and type VII collagen gene expression by TGF- β has been demonstrated. This results in increased expression of procollagen genes and thus contributes to the increased collagen levels in OSMF [14].

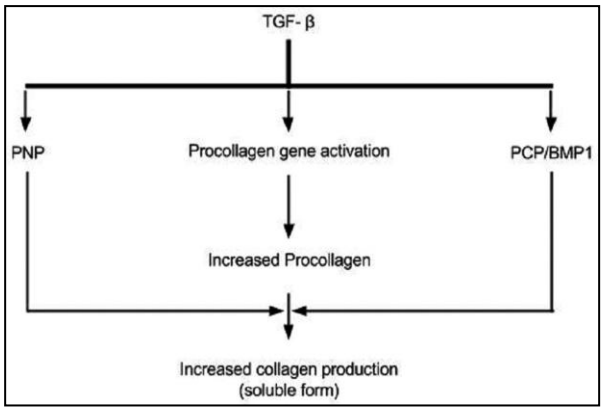


Fig.2: Schematic representation of collagen synthesis regulated by TGF-β

Procollagen proteinases (PNP and PCP) help in processing of procollagen precursors into collagen fibrils, which are soluble. Thus TGF- β modulates both the increased expression of procollagen genes and processing into fibrils by increased levels and activities of PCP and PNP [14].

The end result is increased collagen synthesis.

Collagen cross-linking:

The lysyl oxidase (LOX) enzyme is essential for final processing of collagen fibers into a stabilized covalently cross-linked mature fibrillar form resistant to proteolysis. Its activity leads to the formation of insoluble collagen due to cross-linking. This gives tensile strength and mechanical properties to the fibers which make them resistant to proteolysis.

The LOX is a copper dependent enzyme and the conversion of pro-lysyl oxidase (precursor form) to its active LOX is mediated by BMP1 that takes place in the extracellular matrix. Copper is incorporated in LOX during

its synthesis. Another important co-factor necessary for the cross linking of collagen fibers by LOX is lysine tyrosylquinone (LTQ). It is observed that copper plays an essential role in LTQ stabilization.

Chewing areca nut for 5 – 30 minutes increases the soluble copper levels in the oral fluids which then stimulate fibrogenesis through up-regulation of LOX activity.

The flavonoids present in areca nut also play an essential role in the process of stimulating the cross-linking of the fibers and *in vitro* studies show the presence of catechin in raising the LOX activity.

TGF-β has been considered as an important factor in regulating LOX expression. This could be the elevation of BMP1 by TGF-β which further mediates the bio-synthetic processing of LOX [13].

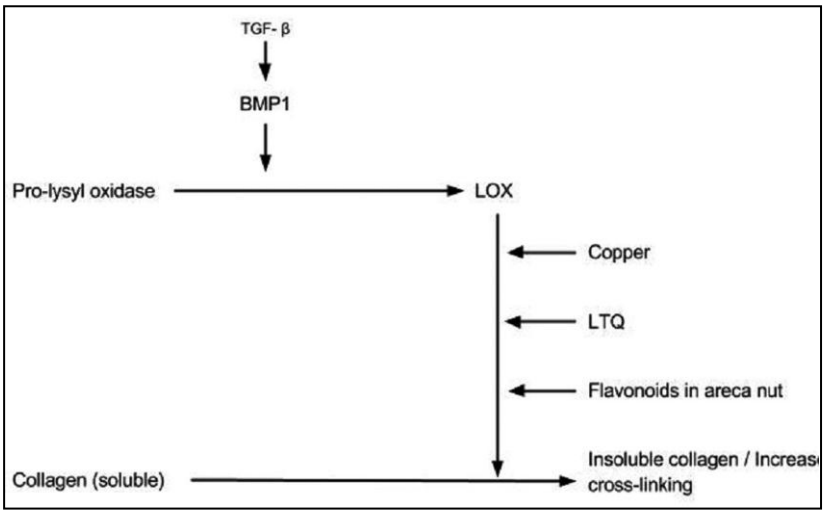


Fig. 3: Schematic representation showing the mechanism involved in increased collagen cross-linking

Thus increase in the LOX activity due to increased BMP and copper levels and presence of flavonoids in areca nut leads to increase in the cross-linking of the collagen fibers, that are resistant to proteolysis.

Arecoline a product of areca nut was also found to elevate Cystatin C mRNA (CST3) and protein expression in a dose-dependent manner. Cystatin C expression was found significantly higher in OSMF specimens and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. Cross-linking between molecules are essential for the tensile strength of collagen fibres. These areas become resistant for collagenase enzymes to attack but can be attacked by a number of other serine and cysteine proteinases. A cysteine proteinase inhibitor encoded by CST3 might contribute to the stabilization of collagen fibrils in OSMF [15].

Decreased collagen degradation:

Collagen degradation by TGF-β is by activation of tissue inhibitor of matrix metalloproteinase gene (TIMPs) and plasminogen activator inhibitor (PAI) gene.

Matrix metalloproteinases (MMPs) comprise a set of structurally related degrading proteases and their main function is tissue remodelling by disruption of extracellular matrix in health and disease. There are many types of MMPs but few MMPs like MMP 1, MMP 8, MMP 13 are referred to as collagenases. The MMPs activities are regulated at the transcriptional level,

activation of the pro-MMPs, and inhibition of endogenous inhibitors under normal physiological conditions [13].

TIMPs are specific inhibitors of MMPs and the local activities in tissues are thus controlled by them. There are studies demonstrating the increased expression of TIMPs in OSMF which are responsible for inhibition of collagenase and decrease in collagen degradation [16,17].

TGF-β activates TIMP gene thus decreasing the collagen degradation by inhibition of the collagenases which are activated. The flavonoids inhibiting the collagenase activity also have been shown [14].

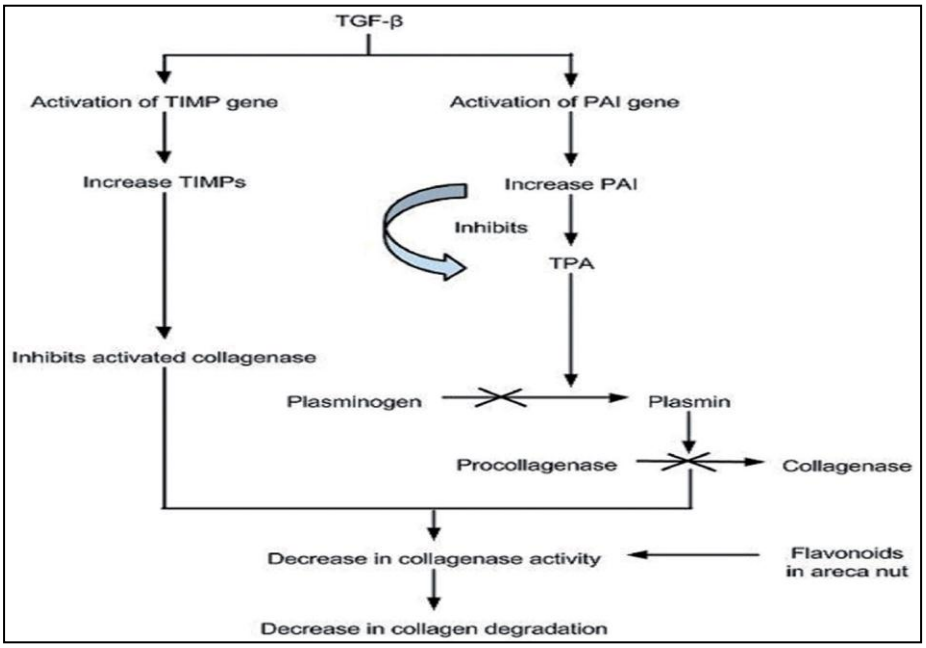


Fig. 4: Schematic representation showing mechanisms involved in decreased collagen degradation

The plasminogen (Plg) activation system, an extra-cellular proteolytic system plays an important role in tissue remodelling. Plasmin is an active serine protease and is generated by proteolytic cleavage of Plg by tissue plasminogen activator (tPA). These activators are regulated by plasminogen activator inhibitors, PAI1 and PAI2.

An essential role is played by plasmin in pro-MMPs activation to active form, and facilitate the degradation of collagen. An increase in PAI1 in OSMF which leads to inhibition of Plg activation process has been shown.

TGF-β plays a very important role in regulation of PAI1 with its stimulatory effect [13].

Hence there is inhibition of existing collagenase and decreased production of active collagenase collectively resulting in decrease in degradation of collagen.

Apart from activation of genes such as TIMPs and PAI, another process which is responsible for decreased collagen degradation is inhibition of collagen phagocytosis. Degradation of collagen by fibroblast phagocytosis has an essential role in physiologic turnover of collagen. *In vitro* culture studies have demonstrated that there is decrease in the collagen phagocytic cells in fibroblasts of OSMF which was thought to be dose dependent with the amount of arecoline/arecaidine [13,18].

Immunologic factors:

Many investigators suggest an autoimmune basis for this disease. It may be because of the presence of HLA A10 and DR3, DR7 antigens found in OSMF patients. Also increased autoantibodies directed toward the thyroid gland, gastric parietal cells, antinuclear antibodies, antismooth muscle antibodies have been seen in OSMF. Humoral immunity may also play a role as increased circulating immune complexes and increased serum levels of IgG and IgA have been found in OSMF patients. Serum-derived antibodies provide further basis for an increase in permeability of the mucosa and exaggerate the already existing pathologic condition. An important link between OSMF and decreased immunity may be the suggested viral origin of the disease (HSV, HPV). Viral lesions show similar immune derangements such as abnormal CD4/CD8 cell ratio as seen in OSMF. Also viral antigen can elicit specific suppressor T-cell response. The resulting immunosuppression allows the spread of antigen and associated epithelial transformation. A defect in the target cell or lysis of viral cell is seen by NK cells in OSMF [19].

Chemical carcinogenesis and OSMF:

As a major component of betel quid preparations, lime causes changes in the oral environment of chewers from neutral to alkaline. In the alkaline condition areca nut ingredients release reactive oxygen species (ROS). Areca phenols at pH > 9.5 undergo auto oxidation to release superoxide radicals and H_2O_2. Also transition metals ions Cu2+, Fe 2+, Fe 3+ promote ROS

production by interacting with areca nut constituents. These ROS are capable of inducing modification in the nucleotide by forming a compound called as 8-hydroxy deoxyguanosine. Mutated initiated cells are formed by this compound during replication. It is also used as a biomarker to predict the attack by chemical carcinogens in the body [19].

CLINICAL FEATURES AND STAGING OF OSMF:

The onset is insidious over a 2-5 year period.

The prodromal/ early symptoms of OSMF:-

This begins with burning sensation in the mouth when consuming spicy food, appearance of blisters especially on the palate, ulcerations or recurrent generalized inflammation of the oral mucosa with excessive salivation, defective gustatory sensation and dryness of' the mouth. They are followed by periods of exacerbation manifested by the appearance of small vesicles in the cheek and palate with intervals which vary from three months to one year. Petechiae which are focal vascular dilatations manifest clinically in the early stages of the disease as part of a vascular response due to hypersensitivity of the oral mucosa towards an external irritant like areca nut products. Petechiae are observed in about 22% of OSMF cases, mostly on the tongue followed by the labial and buccal mucosa with no signs of blood dyscrasias or systemic disorders. A useful clinical test is when on palpation, there is pain in areas where fibrotic bands are developing submucosally.

Histologically, a hyperplastic epithelium, sometimes atrophic with numerous dilated and blood-filled capillaries is seen juxta-epithelially. Inflammatory cells predominantly seen are lymphocytes, plasma cells and eosinophils occasionally. Presence of large numbers of lymphocytes and fibroblasts as well as moderate numbers of plasma cells, suggests' a sustained lymphocytic infiltration in the maintenance of the tissue reaction in OSMF [20].

The advanced OSMF:-

As the disease progresses, the oral mucosa becomes blanched and slightly opaque and there is appearance of white fibrous bands. The buccal mucosa and lips may be affected at an early stage although the palate and the facial pillars were thought to be the areas involved first. The oral mucosa is involved symmetrically mostly although with few exceptions and the fibrous bands in the buccal mucosa run in a vertical direction. The density of the fibrous deposition varies from a slight whitish area on the soft palate causing no symptoms to a dense fibrotic condition causing fixation and shortening or even deviation of the soft palate and uvula. The fibrotic condition of the facial pillars varies from a slight submucosal accumulation in both pillars to a dense fibrosis extending deep into the pillars with the tonsils being strangulated. The presence of this dense fibrosis involving the tissue around the pterygomandibular raphae is that which causes varying degrees of difficulty in mouth opening. This is a factor, which seems to be overlooked by many clinicians while recording the extent of mouth opening because of the

acuteness of oral symptoms at the time of recording. Rarely, the fibrosis spreads to the pharynx and down to the pyriform fossae. A circular band can be felt around the entire *rima oris* (mouth orifice), on palpation and these changes are well appreciated in the lower lip. There has been an impairment of tongue movements observed in patients with advanced OSMF with significant atrophy of the tongue papillae. Stiffening of certain areas of the mucosa occurs with progressing fibrosis leading to difficulty in opening the mouth, inability to whistle or blow and difficulty in swallowing. Once the fibrosis extends to the nasopharynx, the patient may experience referred pain to the ear and a nasal voice as one of the later signs in some patients [20].

Haider SM *et al* (2011) gave the following staging system based on severity of the disease with functional staging measuring inter-incisal opening[21]:

Clinical Staging:

Stage 1: facial bands only

Stage 2: facial and buccal bands

Stage 3: facial and labial bands

Functional Stage:

Stage A: Mouth opening 13 to 20 mm

Stage B: Mouth opening 10 to 11mm

Stage C: Mouth opening

Classification based on Histopathological Features of OSMF:

Histological grading proposed by **Kumar K *et al* (2007)**[2]:

Grade 1: Loose, thick and thin fibers

Grade II: Loose or thick fibers with partial hyalinization

Grade III: Complete hyalinization

MALIGNANT TRANSFORMATION OF OSMF:

Oral submucous fibrosis is a chronic condition of the oral mucosa in Asians, especially Indians. In a cohort conducted by **Wang YY *et al* (2014),** 37 (3.72%) of 994 cases of oral submucous fibrosis transformed into malignancies with a higher transformation rate (1.9%) at an average duration of 37 months, and a shorter mean duration for transformation (52.3 months) [22].

Epidemiological and experimental studies have shown that the process of carcinogenesis is triggered by the production of reactive oxygen species (ROS), which initiate lipid peroxidation (LPO). The extent of oxidative damage caused by ROS can be aggravated by a decrease in the efficiency of the body's antioxidant defense mechanisms. Beta carotene is an essential precursor of vitamin A or retinol and is an excellent antioxidant and radical trapping agent, especially for peroxyl and hydroxyl radicals implicated in the

initiation of a number of cancers. Hence necessary levels of beta-carotene need to be maintained in the blood.

A case control study by **Aggarwal A *et al* (2011)**, clearly indicated a decreased level of beta carotene in Grade I, II and III OSMF. Therefore, beta carotene supplementation in these patients might have direct protective action against cancer. Grade III appeared to have the highest risk of developing cancer, since this group had the minimum level of serum beta carotene, followed in order by Group II and Group I. The study showed that beta carotene played an important role in the pathogenesis of OSMF, and that its level decreased with disease progression and that the degree of oxidative damage in OSMF can be assessed by estimating the levels of serum beta carotene in affected patients, and the underlying antioxidants deficiency can be corrected by dietary supplementation of beta carotene [23].

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor gene and a negative regulator of PI3K/AKT (phosphotidyl inositol-3-kinase/AKT) pathway that controls various cellular processes including proliferation, apoptotic cell death, cell cycle regulation, cell adhesion and migration. Inactivation of PTEN results in proliferation unconditionally and also reduction in apoptosis, thereby predisposing to the development of cancer. Loss of PTEN as a result of somatic mutation or deletion has been reported in a variety of precancers and cancers, including glioblastomas, breast, prostate, endometrial carcinomas, melanomas, head and

neck cancers, and oral squamous cell carcinoma. PTEN has also been responsible for the pathogenesis of fibrotic disorders, such as scleroderma, hepatic fibrosis, kidney, pulmonary, and cardiac fibrosis.

PTEN expression in epithelial cells physiologically regulates the phenotype of these cells, but alteration in normal function of PTEN may contribute to the pathogenesis and exacerbation of fibrosis by inhibiting normal epithelial repair and favouring progression of abnormal fibroblast proliferation [24].

Angadi PV *et al* (2012) proposed the role of PTEN in OSMF based on the downstream targets of PTEN/PI3K pathway studied in OSMF: PTEN downregulation results in:

1) increase in the activity of AKT with effects on downstream targets of PTEN/PI3K pathway that include the alteration of apoptotic factors, such as caspase 9, Bcl-2, p53, and Fas, all of which ultimately increase cell survival;
2) the activation of GSK-3b-b-catenin signalling coupled with decreased p27 leading to increased cell proliferation due to increased cyclin D1 levels; and
3) reduced cell adhesion, increased cell migration, invasion, and epithelial mesenchymal transition by effects on FAK and integrin signaling via ERK and MAPK pathways [24].

Carbonic anhydrase IX (CAIX) is upregulated in many types of human cancers. **Yang JS *et al* (2014)** evaluated plasma levels of CAIX in 191 patients with oral cancer, 30 patients with OSMF, 100 controls and investigated whether plasma CAIX is correlated with the progression of this disease. Patients with oral cancer exhibited significantly higher levels of soluble CAIX compared to controls ($p < 0.001$). In oral cancer patients, plasma levels of CAIX were associated with clinical stages after age adjustment and areca nut chewing ($p < 0.05$). In addition, patients with areca nut chewing had higher CAIX levels than those who have not chewed areca nut. Total carbonic anhydrase activity and CAIX mRNA levels were significantly higher in fibroblasts of oral submucous fibrosis than in fibroblasts of normal buccal mucosa. Moreover, arecoline elevated the expression of CAIX in a dose-dependent manner in fibroblasts of normal buccal mucosa. Therefore, determination of plasma levels of CAIX may be used as a non-invasive method for monitoring the progression of oral cancer and the involvement of betel quid chewing in oral carcinogenesis may be related to increased expression of CAIX [25].

αvβ6 integrin is capable of promoting both tissue fibrosis and carcinoma invasion, **Moutasim KA *et al*(2011)** examined its expression in fibro-epithelial hyperplasia and OSMF. In OSMF, αvβ6 was markedly up-regulated with increase in expression detected in 22 of 41 cases ($p<0.001$). They also investigated the functional role of αvβ6 using oral keratinocyte-

derived cells genetically modified to express high αvβ6 (VB6), and also oral keratinocytes NTERT-immortalized, which express low αvβ6 (OKF6/TERT-1). αvβ6-dependent activation of TGF-β1 showed VB6 cells significantly, which induced the transdifferentiation of oral fibroblasts into myofibroblasts and up-regulated the genes associated with tissue fibrosis. Also arecoline, the major alkaloid of areca nut, up-regulated the expression of keratinocyte αvβ6.Modulated through the M(4) muscarinic acetylcholine receptor, this was suppressed by tropicamide, the M(4) antagonist. The upregulation of arecoline-dependent αvβ6 promoted migration of keratinocyte and induced invasion, raising the possibility that this mechanism may support malignant transformation. Over 80% of OSMF-related oral cancers examined had moderate/high expression of αvβ6 [26].

An immunohistochemical study by **Ranganathan K *et al* (2006)**, was carried out to characterize the cytokeratin (CK) profile in OSMF. Archived tissue blocks of OSMF (n = 50), normal (n = 10) and OC (n = 10) were stained with pancytokeratin (PanCK), high molecular weight cytokeratin (HMWCK), Cytokeratins 18, 14, 8, 5, 4 and 1. Significant difference in the CK staining pattern was seen between normal, OSMF and cancer. Significant changes in OSMF included increase in the intensity of staining for Pancytokeratin and HMWCK, aberrant expression of CK8 and decreased expression of CKs 5 and 14, suggesting their potential as surrogate markers of malignant transformation [27].

Another study by **Nanda KD *et al* (2012)** showed increased expression of CK8 and CK18 in dysplasia, OSMF, and OSCC. The staining pattern and intensity showed variations, with staining intensity in the basal and suprabasal layers for CK8 and CK18 suggesting them as surrogate markers of malignant transformation [28].

Survivin is a recently characterized member of the inhibitor of apoptosis gene family that is differentially expressed in human tumors. Survivin is widely involved in apoptosis, proliferation, embryo development, growth of blood vessels, immune regulation as well as tumor metastasis, which is especially required for tumor maintenance and adequately embodies the nodal role of survivin in cancer networks. Survivin is an important predictive/prognostic parameter of poor outcome in human tumors and will be a diagnostic/therapeutic target in the development of malignant tumors [28].

Zhou S *et al* (2010) examined the phosphorylation of survivin Thr34 in the malignant transformation of OSMF. Survivin Thr34 phosphorylation exhibited significant difference between OSMF and OSCC. During the carcinogenesis of OSMF, the phosphorylation of survivin Thr34 increased gradually, while absence of survivin Thr34 phosphorylation was observed in normal tissue. No relationship was seen between the degree of survivin Thr34 phosphorylation and the stages of OSMF. Phosphorylation of survivin Thr34 was mainly localized in the nucleus. The results indicated the increased phosphorylation level of survivin Thr34 in carcinogenesis of OSMF. Increased

expression of cyclin B1, $p34^{cdc2}$, $p\text{-}p34^{cdc2}$ suggested acceleration of cell proliferation in carcinogenesis of OSMF. Furthermore, p34cdc2 and cyclin B1 formed the activated kinase and phosphorylate survivin on Thr34. The data showed that survivin can be combined with p34cdc2 and transfer ATP to survivin Thr34 in carcinogenesis of OSMF [29,30].

Expression of syndecan-1 is induced during keratinocyte differentiation, and reduced in squamous cell carcinomas (SCCs) and its expression was strongly observed on the keratinocytes of normal oral epithelium and epithelial hyperplasia. Syndecan-1 levels correlate with malignancy in various tissues. The decrease in the levels of mRNA and protein of syndecan-1 has been shown in a wide variety of cancers. An evident correlation has been found between the stage of cancer and its grade and the extent of syndecan loss in SCC (skin, head and neck, oesophageal, or lung), carcinogenesis of lip, oral cancer in dysplasia and SCC of oral cavity, as well as adenocarcinomas (gastric and colorectal). Immunopositivity is lost gradually in epithelial dysplasia, along with the increase in the degree of hyperplasia. In dysplasias, down-regulation of syndecan-1 suggests malignant transformation of the epithelium.

IHC study by **Kamat SS *et al*** showed the results of syndecan-1 expression in the OSMF with dysplasia group were different as 10 cases showed moderate expression and 2 cases showed weak expression. The pattern of syndecan-1 expression in OSMF without dysplasia was comparable

to the normal epithelium. With alterations in expression of syndecan-1 in OSMF, the changes in the cell morphology can be predicted which are related to dysplastic condition at the cellular level. This suggests that it is possible to determine the degree of dysplasia by evaluating intensity of syndecan-1 expression [31].

Mast Cell (MC) activation is a characteristic feature of chronic inflammation that may lead to fibrosis as a result of increased collagen synthesis by fibroblasts. Mast cell tryptase and chymase, the most abundant profibrotic cytokines have been studied in various fibrotic disorders. Neoangiogenesis is required for the growth and spread of tumor. Increased angiogenesis has been associated with neoplastic progression, metastasis and outcome in several studies in numbers of malignancies.

Yadav A *et al* observed a significant increase in the tryptase and chymase positive MCs in OSCC group when compared to OSMF group irrespective of the areas of distribution. It also suggests that upregulation of MCs may play a pivotal role in the progression of tumor during malignant transformation of atrophic epithelium in OSMF. An increase in the number of MC tryptase, a potent proangiogenic factor has been documented similarly in various malignancies including oral cancers. In early OSMF, fibrosis is likely a protective mechanism manifested in the form of excessive collagen deposition, initiated primarily in the connective tissue of the oral mucosa in order to prevent the deeper penetration of carcinogenic substances, while the

transformation of advanced OSMF to cancer is entirely a different issue principally affecting the atrophic epithelium of OSMF if there is persistent carcinogenic insult [32].

The malignant transformation is often associated with genetic changes that in turn is reflected by the altered expression of proteins related to cell cycle, proliferation, and apoptosis. **Ranganathan K *et al* (2011)** and **Humayun S *et al* (2011)** assessed the immunohistochemical expression of p53, Ki67 (MIB), bax and bcl2 in 50 cases of OSMF and ten each of normal and oral squamous cell carcinoma (OSCC). The labelling indices (LI) of OSMF and OSCC were comparable for p53 and Ki67. The p53 LI ranged from 7.9 to 71.9 in OSMF and 65.2 to 85.9 in OSCC, and it ranged from 4.39 to 43.23, 18.35 to 42.33 for Ki67 respectively. The findings of this study indicated that p53, Ki67, and bax exhibited altered expression in OSMF that is different from normal but similar to that in OSCC, and has the potential to be used as surrogate markers of malignant transformation [33,34].

In another study by **Varun BR *et al* (2014)**, it was found that p63 in concert with p53 regulates cell proliferation and differentiation and played a role in potentially malignant and malignant lesions of the oral cavity. Tissue sections of OSCC (n = 20), OSMF (n = 20), leukoplakia (n = 20), and normal oral mucosa (n = 10) were stained with p53 and p63 antibodies by IHC. The significant increase in expression of p53 and p63 proteins in OSCC,

leukoplakia, and OSMF suggested their role as surrogate markers of malignant transformation [35].

Hypoxia-inducible factor (HIF)-1α regulates a wide variety of profibrogenic genes, which have a close association with tissue fibrosis. **Tsai CH *et al* (2014)** conducted an immunohistochemical study using twenty-five OSMF specimens and six normal buccal mucosa. The expression of HIF-1α from fibroblasts cultured from OSMF and normal buccal mucosa was measured by Western blot. Arecoline, a major areca nut alkaloid, was challenged to fibroblasts of normal buccal mucosa to elucidate whether HIF-1α expression could affect by arecoline. Also, the effects of arecoline on plasminogen activator inhibitor (PAI)-1 expression were evaluated in environmental hypoxia. The expression of HIF-1α was significantly higher in OSMF specimens and expressed mainly by epithelial cells, fibroblasts and inflammatory cells. The OSMF derived fibroblasts were found to exhibit higher HIF-1α protein expression than buccal mucosa fibroblasts ($P < 0.05$). HIF-1α protein was found to be upregulated by arecoline in a dose-dependent manner ($P < 0.05$). Arecoline-induced PAI-1 protein expression was increased by hypoxia than normoxic conditions ($P < 0.05$). These results suggested that there is significant upregulation of HIF-1α expression in OSMF tissues from areca quid chewers, implying a potential role as a biomarker for hypoxia in local tissues. The activation of HIF-1α may promote fibrogenesis by an

increased PAI-1 expression and subsequent increase of extracellular matrix production in oral submucosa leading to fibrosis [36].

Anura A *et al* (2014) evaluated the immunohistochemical expression of c-Myc, HIF-1α, VEGF, VEGFRII and CD105 in 58 biopsies of OSMF using computer aided quantification. After digital stain separation of original chromogenic IHC images, the diaminobenzidine reaction pattern was quantified based on intensity and extent of cytoplasmic, nuclear and stromal expression. Assessment of molecular expression proposed that c-Myc and HIF-1α may be used as strong screening markers, VEGF for risk-stratification and VEGFRII and CD105 for prognosis of precancer into oral cancer [37].

Gurudath S *et al* (2012) study was undertaken to estimate and compare erythrocyte superoxide dismutase (E-SOD) and levels of Glutathione peroxidase (GPx) in OSMF, oral leukoplakia and oral cancer patients and age/sex matched healthy subjects, 25 in each group**.** The study had a statistically significant decrease in E-SOD and GPx levels in OSMF, oral leukoplakia compared with the corresponding control group ($p < 0.001$). Also oral cancer group showed a significant ($p<0.001$) decrease statistically in levels of mean E-SOD and GPx when compared to the control group and also the lowest levels among the study groups. This suggests that lower antioxidant enzymes activity in patients with oral cancer might be due to the depletion of

the antioxidant defence system that occurs as the consequence of overwhelming free radicals by the increased levels of lipid peroxides [38].

HIF-1α is associated with the upregulation of growth factors such as vascular endothelial growth factor (VEGF), TGF-β, fibroblast growth factor (FGF), epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF). **Tilakaratne WM *et al* (2008)** used formalin- fixed and frozen samples of OSMF and normal mucosa to investigate the relationship between HIF-1α and epithelial dysplasia using immunohistochemistry and RT-PCR. The data indicated that HIF-1α was upregulated at both protein and mRNA levels in OSMF and the correlation with epithelial dysplasia was statistically significant ($P < 0.001$). Increased expression of HIF-1α in fibroblasts and epithelial cells supported that hypoxia plays a role in progression of fibrosis in OSMF once there is initiation of the disease process by arecoline in betel quid[3].

Pandiar D *et al* (2014) conducted a study to evaluate CD34 and basic fibroblast growth factor (bFGF) immunoreactivity in different histological grades of OSMF for the role of microvasculature, so that the epithelial atrophy and resultant malignant transformation seen in the advanced stages might be elucidated. A total of 30 cases of OSMF were included in the study and mean vascular density (MVD) was calculated using CD34 and bFGF. 5 cases of OSMF with dysplasia and 2 cases of OSMF turning malignant have also been added in the study. Mean vascular density was found to decrease significantly

as the diseases advanced. Further, there was increase in vascularity significantly in cases of OSMF turning towards malignancy. The observation of decreased vascularity in different stages of OSMF and the relationship of epithelial thickness significant with the stages, reiterated the prevailing concept that lack of perfusion leads to epithelial atrophy, which may later undergo dysplastic changes and turn malignant [39].

ROLE OF HIF - 2α IN TUMOR MODELS:

The cellular response to hypoxia includes the transcription of genes induced by hypoxia- inducible factor-1 (HIF-1) which are involved in diverse processes such as glycolysis and angiogenesis. An alternative dimerization partner for ARNT (Aryl hydrocarbon receptor nuclear translocator**)**, which also transactivates genes via HIF DNA recognition sites, has been termed endothelial PAS domain protein 1 (EPAS-1), HIF-1α-like factor (HLF), and mouse HIF related factor (HRF). In accordance to the functional homology with HIF- 1 alpha, it is called HIF -2 alpha.

In normal tissues:

Distribution of both HIF-1α and HIF-2α within human tissue has primarily been of mRNA. *In situ* studies have found the prevalence of HIF-1α RNA in all tissues other than peripheral blood leukocytes, and expression of HIF-2α RNA to be high in vascular tissues such as placenta, lung, heart, and kidney.

Talks KL *et al* (2000) investigated the distribution of HIF-1α and HIF-2α in normal and pathological tissues by immunohistochemistry using monoclonal antibodies (mAb). A new mAb to detect HIF-1α, was designated 122, and validated mAb 190b to HIF-2α was used**.** In examining a majority of solid tumors, including colon, ovarian, pancreatic, prostate, bladder, brain, breast and renal carcinomas, nuclear expression of HIF-1α and -2α was seen in varying subsets of the tumor cells. A strong expression of HIF-2α was also seen in subsets of tumor-associated macrophages, sometimes in the absence of any tumor cell expression. Staining was observed in other stromal cells within the tumors less frequently and adjacent to tumor margins in normal tissue. In contrast, in normal tissue neither molecule was detectable except within subsets of bone marrow macrophages, where HIF-2α was strongly expressed[7,40].

RNA *in situ* hybridization on mouse embryos revealed that expression of HIF2α is more restricted, and abundant particularly in blood vessels. In a direct comparison of HIF1α and HIF2α function in a (Kirsten rat sarcoma viral oncogene homolog) KRAS-driven lung tumor model, by **Keith B *et al* (2012),** deletion of HIF1α surprisingly had little effect on tumor burden and progression, whereas loss of HIF2α actually increased tumor growth and progression. Overexpression of a stabilized HIF2α protein in the identical KRAS lung tumor model also promoted tumor invasion and angiogenesis by increasing expression of vascular endothelial growth factor (VEGF) and

SNAIL45, respectively. The overexpression or deletion of HIF2α can promote tumor growth in the same tumor context, albeit by different mechanisms, suggests that targeting of HIFα subunits effectively in cancer treatment may be complicated. Growth of mouse liver hemangiomas which are pVHL-deficient was similarly shown to be specifically dependent on HIF2α, but not HIF1α [41].

The expression of HIF-2 alpha protein has been observed postnatally in a number of cell populations in different tissues of mice treated with hypoxia or hypoxia-mimetic agents. In addition to being present in endothelial cells, HIF-2 alpha mRNA has also been detected in liver hepatocytes, epithelial cells of the intestinal lumen, kidney fibroblasts, heart myocytes and interstitial cells, pancreatic interstitial cells, and lung type II pneumocytes. Almost all transformed cell lines exhibit HIF-2 alpha expression in contrast to *in vivo* restricted expression patterns. Moreover, HIF-2 alpha expression in tumor vascular cells, parenchymal cells, and infiltrating macrophages has also been shown [5].

In mouse xenograft models, expression of HIF2α (and not HIF1α) is crucial for growth of clear cell renal cell carcinoma (ccRCC) and neuroblastoma tumors. HIF2α inhibition promotes tumor cell death and, in contrast to HIF1α, enhances the response to radiation treatment. A study by **Bertout JA *et al* (2009)** on culture inhibiting HIF2α expression augments p53 activity, increased apoptosis, and reduced clonogenic survival of irradiated and non-irradiated cells. Moreover, inhibition of HIF2α promoted p53-

mediated responses by disrupting the homeostasis of cellular redox, thereby permitting reactive oxygen species (ROS) accumulation and DNA damage. These results correlated with altered p53 phosphorylation and target gene expression in untreated human tumor samples and showed that HIF2α likely contributes to tumor cell survival including during radiation therapy [40,42].

HIF – 2α in colon cancer:

Imamura T *et al* (2009) studied that SW480 colon cancer cells with stable expression of siRNA to HIF-1α or HIF-2α or both were established. Lower rates of proliferation and migration were displayed by HIF-1α-deficient cells, but HIF-2α-deficient cells showed enhanced anchorage independent growth in a soft agar assay. Xenograft studies revealed that deficiency of HIF-1α prevented overall tumor growth, whereas HIF-2α deficiency stimulated tumor growth. Expression of HIF-1α in human colon cancer tissues, and HIF-2α to a lesser extent, was linked to upregulation of VEGF and tumor angiogenesis. However, loss of HIF-2α expression but not HIF-1α was strongly correlated with advanced tumor stage [43].

HIF - 2α in breast cancer:

Leek RD *et al* (2002) examined whether the involvement of HIF-2α in angiogenic activation of tumor-associated macrophages (TAM) by correlating its expression with tumor microvessel density as a marker of angiogenesis, and other tumor variables, in human primary invasive breast carcinoma series. A

correlation was found between increased TAM HIF-2α and increased tumor vascularity ($P < 0.0001$), as well as high tumor grade ($P = 0.007$). The relation of HIF-2α expression to an oxygen-dependent pathway of angiogenesis was also studied, and an inverse relationship was found between TAM HIF-2α and expression of tumor thymidine phosphorylase ($P = 0.02$). These results suggested that TAM HIF-2 signalling may be a useful target for future anti-angiogenic strategies but show that tumors use both oxygen-dependent and oxygen deficiency regulated pathways for angiogenesis [44].

Differential expression in breast epithelial cells:

Pahlman S *et al*, stated that HIF-2α can accumulate and get activated in response to hypoxic stress. This was explained by their immunohistochemical study of the oxygen sensitive HIF-alpha subunits using tumors from a transgenic mouse mammary tumor model injected with Hypoxyprobe as reference tissue. The mammary gland of mouse was studied during the development, lactation and involution stages. Since hypoxia affects normal epithelial differentiation, HIFs were considered important for normal breast epithelial development and regeneration as well as cancer initiation and progression. It has been shown in breast epithelium that HIF-2α is associated with breast cancer metastasis and poor patient survival. It has also been reported that hypoxia and the transcriptional activity are linked to a state of loss of polarisation and a cancer like phenotype in primary human breast epithelial cells [45].

In the epithelial cells of the breast, HIF-2 alpha was not found to be expressed at the developmental stage but rather restricted to a subpopulation of luminal epithelial cells during lactation phase when epithelial cells in the mammary glands undergo full differentiation and the metabolism is extremely high as energy-rich milk is produced. Due to the shear stress from the milk-filled ducts and metabolic stress due to increased demand for nutrients during milk production, there is an increased expression of HIF-2α which is found to be non-hypoxic. During the involution stage, infiltrating macrophages were among the HIF-2α expressing cells which is most interesting, since this stage involves numerous processes pivotal to carcinogenesis and cancer progression. The divergent role played by HIF-2α at these stages suggest that their regulation is not merely a function of oxygen availability in the mammary epithelium [45].

HIF -2α in digestive system cancers:

HIF-2α was shown to mediate microRNA-210 and c-Myc to participate in neoplasms. MicroRNA-210 is a direct transcriptional target of HIF-2α and its upregulation has led to a switch from Mnt to c-Myc expression during cholestatic cholangiocarcinogenesis *in vivo*. The function of HIF-2α appears to be dependent on cell-type. The different expression patterns and levels of HIF-2 as well as cell-specific cofactors may affect its activity. Poorer survival of HIF-2α and wild-type TP53 was associated with carbonic anhydrase 9 (CA9) stromal-positive colorectal adenocarcinomas. In addition,

poor prognosis is seen of tumors expressing HIF-2α or CA9 in their stroma in wildtype TP53 tumors compared with mutant malignancies. Under hypoxia, HIF-2α is involved in metastasis and invasion of gastric cancer cells, in a mechanism involving the JNK signaling pathway. In primary colorectal cancer, overexpression of HIF-2α together with BNIP3 was linked to local invasion and lymph node metastasis [6,43].

HIF- 2α in hepatocellular carcinoma:

In an immunohistochemical study on paraffin-embedded sections from 97 patients with HCC, **Bangoura G *et al* (2004)** investigated the expression of HIF-2α/EPAS1. Furthermore, to confirm that HIF-2α/EPAS1 in HCC tissues also correlated with angiogenesis, a parallel immunohistochemical study of vascular endothelial growth factor (VEGF) was performed on these 97 cases. In 50 of 97 cases (51.6%), HIF-2α/EPAS1 could be detected, including 19 weakly positive (19.8%), and 31 strongly positive (31.1%), the remaining 47 cases were negative (48.4%). HIF-2α/EPAS1 expression was significantly correlated with tumor size, capsule infiltration, invasion of portal vein and necrosis. A parallel immunohistochemical analysis of VEGF demonstrated its positive correlation with capsule infiltration, invasion of portal vein, and HIF-2α/EPAS1 overexpression, which supported the correlation of tumor angiogenesis with HIF-2α/ EPAS1 up-regulation [46].

HIF – 2α IN OSMF AND ITS MALIGNANT TRANSFORMATION:

Vascular endothelial growth factor (VEGF) is responsible for angiogenesis, which has been a key event in carcinogenesis. Further, in microscopical sections of OSMF associated with epithelial dysplasia, the presence of increased number of blood vessels has been observed [3].

Overexpression of a stabilized HIF2α protein in the identical KRAS lung tumor model also promoted tumor angiogenesis and invasion and by elevating expression of vascular endothelial growth factor (VEGF) and SNAIL45, respectively [41].

HIF2α has also been reported to regulate antioxidants such as superoxide dismutase 1 (SOD1), SOD2, catalase and glutathione peroxidise 1 in embryos which are developing and neonates. However, in RGG cells, HIF2α instead decreases accumulation of ROS by regulating the expression of distinct antioxidant enzymes (hemeoxygenase 1, ceruloplasmin, glutathione peroxidase 8, and peroxiredoxin 3).

Loss of HIF2α function in endothelial cells reduced expression of ephrin A152, angiopoietin 2 (ANG2)53 and delta-like ligand 4 (DLL4) which correlated with unproductive sprouting and aberrant vessel remodelling and xenograft tumor growth [41].

Results

SAMPLE CHARACTERISTICS:

The study population comprised of 51 cases taken from the archival blocks. They were categorized into four groups. Group 1 (n =11) comprising of oral submucous fibrosis specimen (OSMF) samples, Group 2 (n = 15) comprising of oral squamous cell carcinoma associated with areca nut habit samples, Group 3 comprising of (n =15) oral squamous cell carcinoma without areca nut habit specimen samples and Group 4 comprising of (n = 10) clinically normal appearing mucosa specimen samples. All the samples were analyzed for the immunoreactivity of HIF – 2α stain.

Distribution of age in the study groups (Table 1 & Graph 1):

The distribution of the age of patients were divided into 3 groups: 20-40 years, 41-60 years and those above 61 years of age. Group 1 consisted of 4 (36%) cases in the age group 20-40 years, 6 (54%) cases in the age group of 41-60 and 1(9%) case above 61 years. Group 2 consisted of 6 (40%) cases in 20-40 years, 7 (47%) cases in 41-60 years and 2 (13%) cases above 61 years. Group 3 consisted of 1 (7%) case in 20-40 years, 10 (67%) cases in 41-60 years and 4 (27%) cases of above 61 years. Group 4 consisted of 9 (90%) cases in 20-40 years and 1(10%) case in the age group of 41-60 (p=0.006).

Distribution of gender in the study groups (Table 2 & Graph 2):

In group 1, 9 (82%) were males and there were 2 (18%) females. In group 2, 15 (100%) were males. In group 3, 10 (67%) were males and 5 (33%) were females. In group 4, 6 (60%) were males and 4 (40%) were females. (p=0.059).

Distribution of habits in the study groups (Table 3 & Graph 3):

Based on the prevalence of habits in the study groups, they were categorized in to four groups. They were those with the habit of chewing areca nut (either alone or in combination with tobacco and alcohol), tobacco (smokeless or smoking form) only, tobacco along with consumption of alcoholic beverages and those without any habits. In group 1, 9 (82%) had the habit of chewing areca nut (either alone or in combination with tobacco and alcohol), 2 (18%) had the habit of using tobacco along with alcohol. In group 2, all the 15 (100%) had the habit of chewing areca nut (either alone or in combination with tobacco and alcohol). In Group 3, 2 (13%) had the habit of chewing tobacco and smoking, 13 (87%) had no habits. In group 4 (control group), all the 10 (100%) had no habits (p=0.00).

Distribution of site in the study groups (Table 4 & Graph 4):

In group 1, all 11 (100%) cases were from buccal mucosa. In group 2, 6 (40%) cases were from lateral border of tongue, 7 (47%) cases from buccal mucosa, 1 (7%) was from the buccal vestibule and 1 (7%) from the gingiva.

In group 3, 7 (47%) cases were from the lateral border of tongue, 2 (13%) from the buccal mucosa, buccal vestibule and palate, 1 (7%) from gingiva and commissure of mouth. In group 4, 2 (20%) cases were from the gingiva, 8 (80%) incisional biopsies were from the retro-molar region. (p=0.00)

Distribution of staining of HIF-2α in the study groups (Table 5 & Graph 5):

Of the total number of cases subjected to HIF-2α staining, in group 1, 11 (100%) cases and in group 2, 15 (100%) cases had the expression. In group 3, 13 (87%) expressed HIF-2α and 2 (13%) did not express. In group 4, 9 (90%) cases expressed and 1 (10%) case did not show the expression. (p=0.329)

The following parameters were used to evaluate HIF-2α staining in all the 4 groups:

- Staining intensity
- Staining pattern
- Percentage of cells stained
- Tissue localisation of the stain

TISSUE LOCALIZATION OF THE STAIN (Table 6 and Graph 6):

The HIF-2α staining was seen in the basal and supra basal layers of the epithelium and the connective tissue (RBCs, blood vessels and muscle fibres). In group 1, 2 (18%) cases showed suprabasal staining, 3 (27%) cases showed connective tissue staining and 6 (54%) cases showed basal and suprabasal layers and connective tissue staining. In group 2, 5 (33%) cases showed supra-basal staining, 3 (20%) cases showed both basal and suprabasal staining and 7 (47%) cases showed basal and suprabasal layers and connective tissue staining. In group 3, 2 (13%) cases showed no expression, 1 (7%) case showed suprabasal staining, 3 (20%) cases showed basal and suprabasal staining, 1 (7%) case showed suprabasal along with connective tissue and 8 (53%) cases showed basal and suprabasal layers and connective tissue staining. In group 4, 1(10%) case showed no staining, suprabasal staining alone, 5 (50%) cases showed both basal and suprabasal staining, 1 (10%) case showed suprabasal along with connective tissue staining and 2 (20%) cases showed basal and suprabasal and connective tissue staining. (p value = 0.023)

STAINING INTENSITY

Comparison of overall HIF-2α intensity between the study groups (Table 7 & Graph 7):

On comparing the HIF-2α intensity between the study groups, Group 1 showed 5 (45%) cases & 6 (54%) cases with mild and moderate staining

respectively. Group 2 showed 8 (54%), 5 (33%) and 2 (13%) cases with mild, moderate and intense staining respectively. In Group 3, 7 (47%) and 6 (40%) cases showed mild and moderate staining respectively with 2 (13%) showing no expression. In group 4, 6 (60%) and 3 (30%) cases showed mild and moderate staining respectively and 1(10%) case showed no expression ($p = 0.406$)

Comparison of basal layer intensity between the study groups (Table 8 & Graph 8)

On analyzing the basal layer intensity of HIF-2α in all the four groups, in group 1, 6 (54%) cases showed mild staining and 5 (45%) showed no expression. In group 2, 5 (33%) cases did not take up staining in the basal layer, 7 (47%), 2 (13%) and 1 (7%) cases showed mild, moderate and intense staining respectively. In group 3, 4 (27%) cases did not take up the stain, remaining 10 (67%) and 1 (7%) cases showed mild and moderate staining respectively. In group 4, 3 (30%) cases did not take up the staining in this layer and remaining 7 (70%) exhibited mild staining ($p =0.682$).

Comparison of supra basal layer intensity between the study groups (Table 9 & Graph 9):

On analyzing the supra basal layer intensity of HIF-2α in all the four groups, in group 1, 3 (27%) cases did not take up the stain, 3 (27%) and 5 (45%) cases showed mild and moderate staining respectively. In Group 2,

7 (47%), 5 (33%) and 3 (20%) cases showed mild, moderate and intense staining respectively. In group 3, 2 (33%) cases did not take up staining and remaining 7 (47%), 5 (33%) and 1 (7%) cases showed mild, moderate and intense staining respectively. In group 4, 1 (10%) case did not take up the staining in the suprabasal layer and remaining 7 (70%), 1(10%) and 1(10%) cases showed mild, moderate and intense staining ($p = 0.268$)**.**

Comparison of connective tissue intensity between the study groups (Table 10 & Graph 10):

On analyzing the connective tissue intensity of HIF-2α in all the four groups, in group 1, 2 (18%) cases did not take up the stain, 5 (45%) and 4 (36%) cases showed mild and moderate staining respectively. In Group 2, 8 (53%) cases showed no staining, 2 (13%), 4 (27%) and 1 (7%) showed mild, moderate and intense staining respectively. In group 3, 6 (40%) cases did not take up staining and remaining 6 (40%), 2 (13%) and 1(7%) cases showed mild, moderate and intense staining respectively. In group 4, 7 (70%) cases did not take up the staining in the connective tissue and remaining 2 (20%) and 1 (10%) cases showed mild and moderate staining ($p = 0.319$)**.**

STAINING PATTERN:

Comparison of pattern of staining of HIF-2α among the study groups (Table 11 & Graph 11):

The staining pattern of HIF-2 alpha is divided into no expression, cytoplasmic and combined cytoplasmic and nuclear expression. In group 1, 6 (54%) cases exhibited cytoplasmic staining, 2 (18%) cases exhibited both cytoplasmic and nuclear staining and 3 (27%) cases did not take up either stain. In group 2, 8 (53%) cases exhibited cytoplasmic staining, 7 (47%) cases showed both cytoplasmic and nuclear staining. In group 3, 2 (13%) did not take up the stain, 10 (67%) cases showed cytoplasmic staining and 3 (20%) cases exhibited both cytoplasmic and nuclear staining. In group 4, 4 (40%) cases showed cytoplasmic staining and 5 (50%) cases showed both nuclear and cytoplasmic staining and 1 (10%) case did not take up the stain. There were no cases with nuclear staining alone (p value=0.234).

PERCENTAGE OF POSITIVE CELLS:

Nuclear labelling index of HIF-2 alpha positive cells among the study groups (Table 12 & Graph 12):

The percentage of HIF-2 alpha positive cells were divided into four groups namely <20%, 20-50%, >50% and negative. In group 1, 9 (82%) cases exhibited no expression and 2 (18%) cases had 20-50% positively stained cells. In group 2, 1 (7%) case had less than 20%, 5 (33%) had 20-50% and

1 (7%) case had more than 50% of positively stained cells and 8 (53%) negative cells. In group 3, 12 (80%) had negative cells, 1 (7%) had 20-50% and 2 (13%) had more than 50% of positive cells. In group 4, 3 (30%) had 20-50% and 1 (10%) had more than 50% of positive cells and 6 (60%) had negative cells (p=0.534).

Comparison of the mean labelling index of HIF-2α between the study groups (Table 13):

The mean labelling index of HIF-2α staining between the groups 1 & 2, groups 1 & 3, groups 1 & 4, groups 2 & 3, groups 2 & 4 and groups 3 & 4 showed no significant difference (p value=0.282).

Distribution of HIF-2α positive stained cells with histopathologic grading of oral squamous cell carcinoma (Group 2) (Table: 14, Graph: 14)

The percentage of positive stained cells were divided into four groups namely no expression, <20%, 20-50% and >50%. In well differentiated SCC, 4 (44%) cases showed no expression, 4 (44%) cases had 20-50% of positivity and 1 (11%) case had more than 50% of positive cells. In moderately differentiated SCC, 4 (67%) cases showed no expression, 1 (17%) case had <20%, 1 (17%) case had 20-50% of positive stained cells (p=0.343).

Distribution of HIF-2α positive stained cells with histopathologic grading of oral squamous cell carcinoma (Group 3) (Table: 15, Graph: 15)

The percentage of positive stained cells were divided into four groups namely no expression, <10%, 10-50% and >50%. In well differentiated OSCC, 1 (10%) case had 20-50% of positive cells and 2 (20%) cases had more than 50% of cells positive and 7 (70%) cases did not take up the stain. In moderately differentiated OSCC, all 2(100%) cases and in poorly differentiated OSCC, all 3 (100%) cases did not take up the stain (p=0.759).

Comparison of HIF-2α intensity between the grades of OSCC (Group 2) (N=15) (Table: 16, Graph 16)

The grades of OSCC were divided into well and moderately differentiated OSCC. In well differentiated OSCC, 5 (55%) cases showed mild intensity, 3 (33%) cases showed moderate intensity and 1 (11%) case showed intense intensity. In moderately differentiated OSCC, 3 (50%) cases showed mild intensity, 2 (33%) cases showed moderate intensity and 1 (17%) case showed intense intensity (p value=0.949).

Comparison of HIF-2α intensity between the grades of OSCC (Group 3) (N=15) (Table: 17, Graph 17)

The grades of OSCC were divided into well, moderately and poorly differentiated OSCC. In well differentiated OSCC, 7 (70%) cases showed mild intensity and 3 (30%) cases showed moderate intensity. In moderately

differentiated OSCC, 1 (50%) case did not take up the stain and 1 (50%) case showed moderate intensity. In poorly differentiated OSCC, 1 (33%) case was negative and 2 (67%) showed moderate intensity (p value=0.072).

Inter-comparison of HIF-2α intensity between Group 1 and Group 2 (Table: 18)

Group 1 showed 5 (45%) cases mild and 6 (54%) cases moderate intensity. Group 2 showed 8 (54%) cases mild, 5 (33%) cases moderate and 2 (13%) cases with intense intensity (p value=0.329).

Inter-comparison of HIF-2α intensity between Group 1 and Group 3 (Table: 19)

Group 1 showed 5 (45%) cases mild and 6 (54%) cases with moderate intensity. Group 3 showed 2 (13%) cases with negative expression, 7 (47%) cases mild and 6 (40%) cases moderate intensity (p value=0.415).

Inter-comparison of HIF-2α intensity between Group 1 and Group 4 (Table: 20)

Group 1 showed 5 (45%) cases mild and 6 (54%) cases moderate intensity. In group 4, 1 (10%) case did not take up the stain and remaining 6 (60%) and 3 (30%) cases showed mild and moderate intensity respectively (p value=0.359).

Inter-comparison of HIF-2α intensity between Group 2 and Group 3 (Table: 21)

Group 2 showed 8 (54%) cases mild, 5 (33%) cases moderate, 2 (13%) cases with intense intensity. In group 3, 2 (13%) cases did not take up the stain and remaining 7 (47%) and 6 (40%) cases showed mild and moderate intensity respectively (p value=0.245).

Inter-comparison of HIF-2α intensity between Group 2 and Group 4 (Table: 22)

Group 2 showed 8 (54%) cases mild, 5 (33%) cases moderate, 2 (13%) cases with intense intensity. In group 4, 1 (10%) case did not take up the stain and remaining 6 (60%) and 3 (30%) cases showed mild and moderate intensity respectively (p value=0.407).

Inter-comparison of HIF-2α intensity between Group 3 and Group 4: (Table: 23)

Group 3 showed 2 (13%) cases with negative expression, 7 (47%) cases mild and 6 (40%) cases moderate intensity. In group 4, 1 (10%) case did not take up the stain and remaining 6 (60%) and 3 (30%) cases showed mild and moderate intensity respectively (p value=0.808).

Kappa value:

The overall Kappa value for the inter observer variation is 0.85.

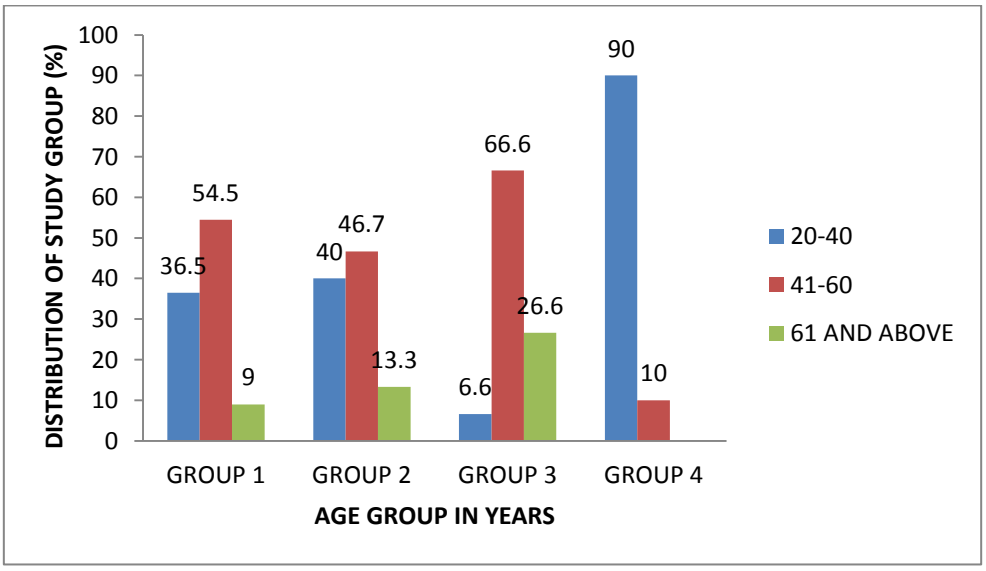
Tables and Graphs

TABLE 1: DISTRIBUTION OF AGE IN STUDY GROUPS (N=51)

AGE GROUPS IN YEARS	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
20-40	4(36.5%)	6(40%)	1(6.6%)	9(90%)	0.006*
41-60	6(54.5%)	7(46.7%)	10(66.6%)	1(10%)	
61 AND ABOVE	1(9.0%)	2(13.3%)	4(26.6%)	0(0%)	

***p Value ≤ 0.05 is significant**

GRAPH 1: DISTRIBUTION OF AGE IN STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

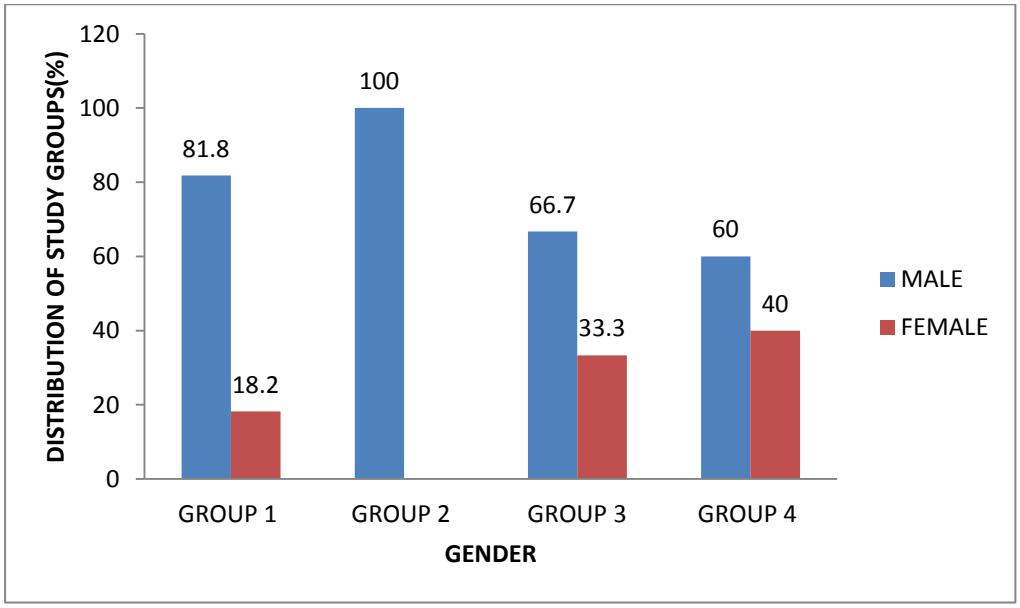
GROUP IV – Normal mucosa (n=10)

TABLE 2: DISTRIBUTION OF GENDER IN STUDY GROUPS (N=51)

GENDER	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
MALE	9(81.8%)	15(100%)	10(66.7%)	6(60%)	0.059*
FEMALE	2(18.2%)	0(0%)	5(33.3%)	4(40%)	

***p Value ≤ 0.05 is significant**

GRAPH 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

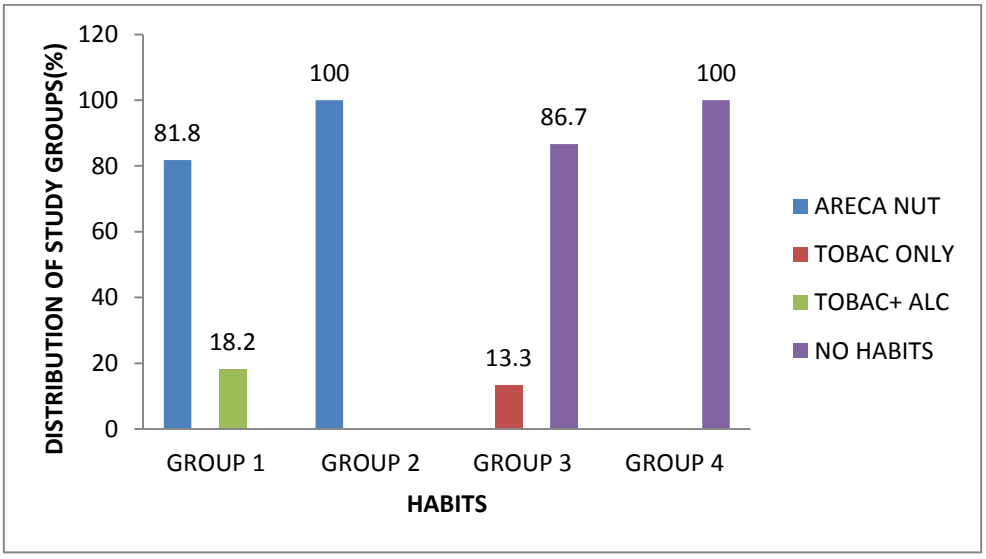
TABLE 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS

(N=51)

HABITS	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
ARECA NUT	9(81.8%)	15(100%)	0(0%)	0(0%)	0.000*
TOBACCO ONLY	0(0%)	0(0%)	2(13.3%)	0(0%)	
TOBACCO + ALCOHOL	2(18.2%)	0(0%)	0(0%)	0(0%)	
NO HABITS	0(0%)	0(0%)	13(86.7%)	10(100%)	

***p Value ≤ 0.05 is significant**

GRAPH 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

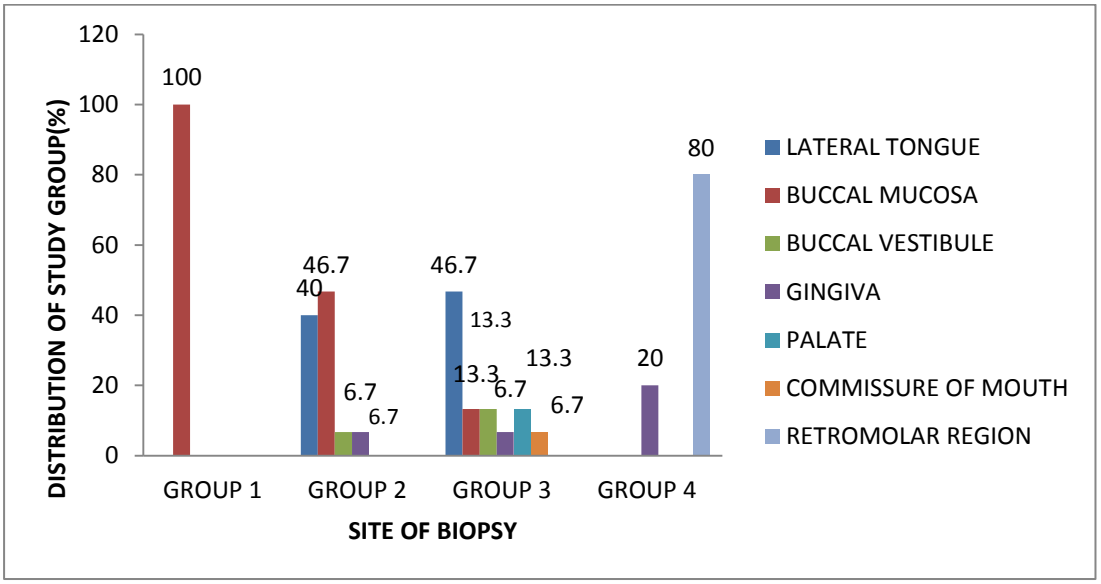
GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 4: DISTRIBUTION OF SITE OF BIOPSY IN THE STUDY GROUPS (N= 51)

SITE OF BIOPSY	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
LATERAL TONGUE	0(0%)	6(40%)	7(46.7%)	0(0%)	0.594
BUCCAL MUCOSA	11(100%)	7(46.7%)	2(13.3%)	0(0%)	
BUCCAL VESTIBULE	0(0%)	1(6.7%)	2(13.3%)	0(0%)	
GINGIVA	0(0%)	1(6.7%)	1(6.7%)	2(20%)	
PALATE	0(0%)	0(0%)	2(13.3%)	0(0%)	
COMMISSURE OF MOUTH	0(0%)	0(0%)	1(6.7%)	0(0%)	
RETROMOLAR REGION	0(0%)	0(0%)	0(0%)	8(80%)	

GRAPH 4: DISTRIBUTION OF SITE OF BIOPSY IN THE STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

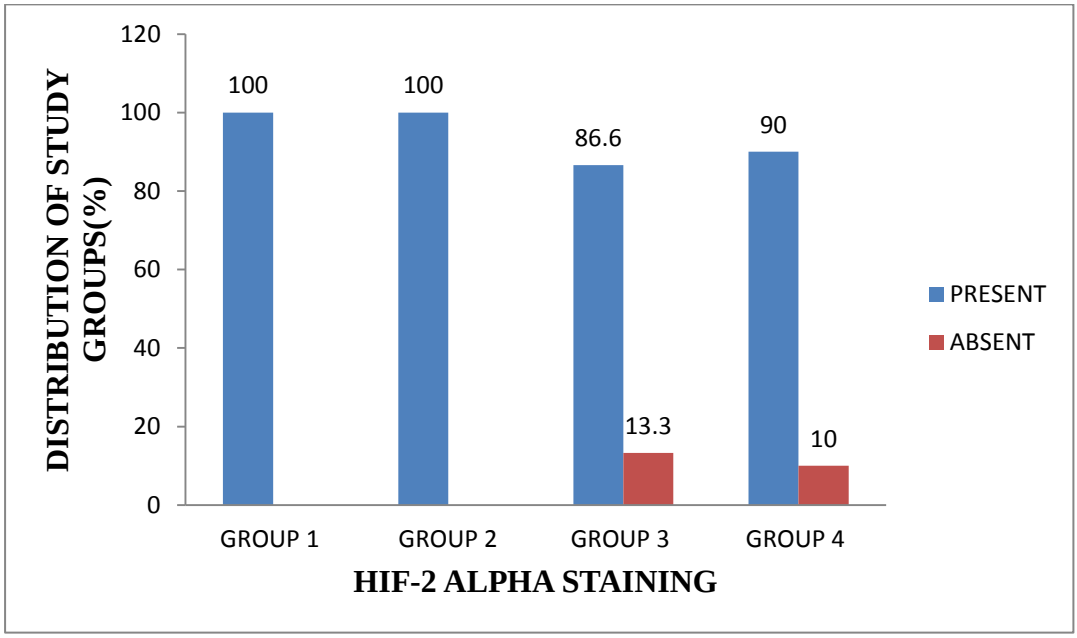
GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 5: DISTRIBUTION OF STAINING OF HIF-2 ALPHA AMONG THE STUDY GROUPS (N=51)

HIF - 2 ALPHA STAINING	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
PRESENT	11(100%)	15(100%)	13(86.6%)	9(90%)	0.329
ABSENT	0(0%)	0(0%)	2(13.3%)	1(10%)	

GRAPH 5: DISTRIBUTION OF STAINING OF HIF-2 ALPHA IN THE STUDY GROUPS



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

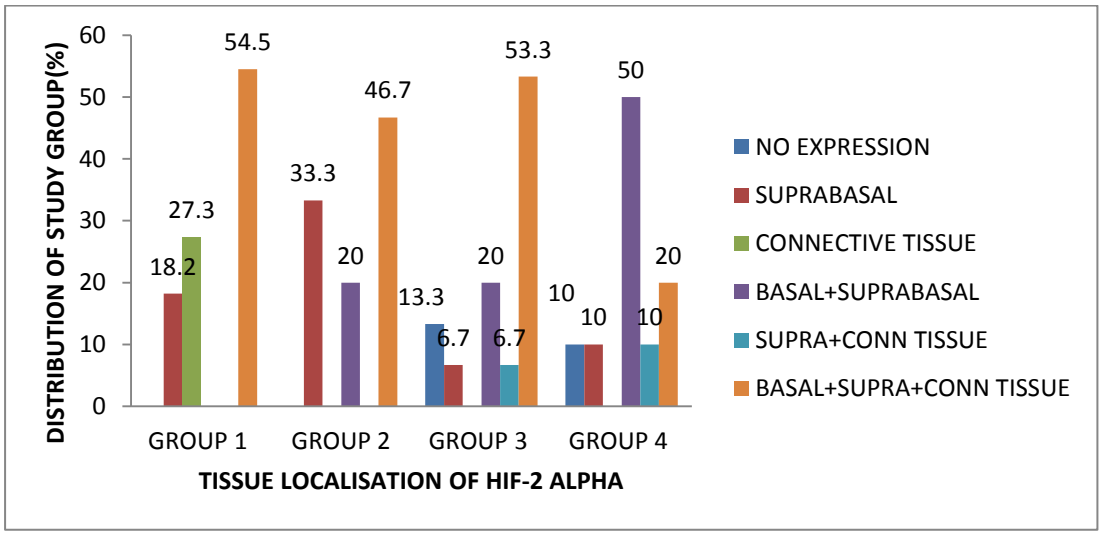
GROUP IV – Normal mucosa (n=10)

TABLE: 6 TISSUE LOCALISATION IN HIF-2 ALPHA POSITIVE STAINED CELLS IN THE STUDY GROUPS (N=51)

TISSUE LOCALISATION	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	0(0%)	0(0%)	2(13.3%)	1(10%)	0.023*
SUPRABASAL	2(18.2%)	5(33.3%)	1(6.7%)	1(10%)	
CONNECTIVE TISSUE	3(27.3%)	0(0%)	0(0%)	0(0%)	
BASAL+SUPRABASAL	0(0%)	3(20%)	3(20%)	5(50%)	
SUPRABASAL+ CONNECTIVE TISSUE	0(0%)	0(0%)	1(6.7%)	1(10%)	
BASAL+SUPRABASAL+ CONNECTIVE TISSUE	6(54.5%)	7(46.7%)	8(53.3%)	2(20%)	

***p Value ≤ 0.05 is significant**

GRAPH: 6 TISSUE LOCALISATION IN HIF-2 ALPHA POSITIVE STAINED CELLS IN THE STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11) **GROUP IV – Normal mucosa (n=10)**

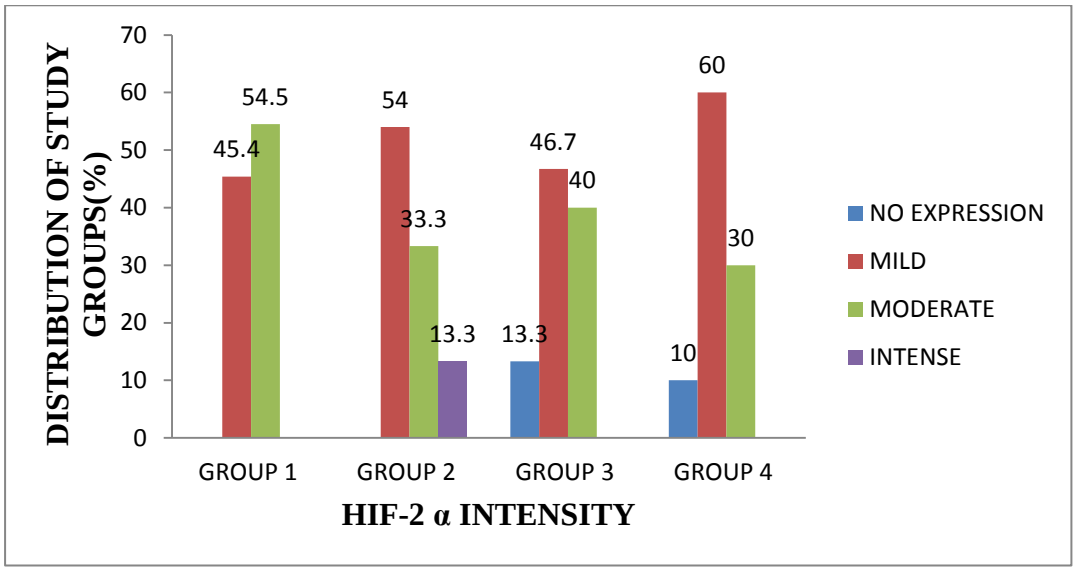
GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

TABLE 7: COMPARISON OF HIF-2 ALPHA INTENSITY BETWEEN THE STUDY GROUPS (N=51)

HIF-2 ALPHA INTENSITY	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	0(0.0%)	0(0.0%)	2(13.3%)	1(10%)	0.406
MILD	5(45.4%)	8(54%)	7(46.7%)	6(60%)	
MODERATE	6(54.5%)	5(33.3%)	6(40%)	3(30%)	
INTENSE	0(0%)	2(13.3%)	0(0%)	0(0%)	

GRAPH 7: DISTRIBUTION OF HIF-2 ALPHA INTENSITY BETWEEN THE STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

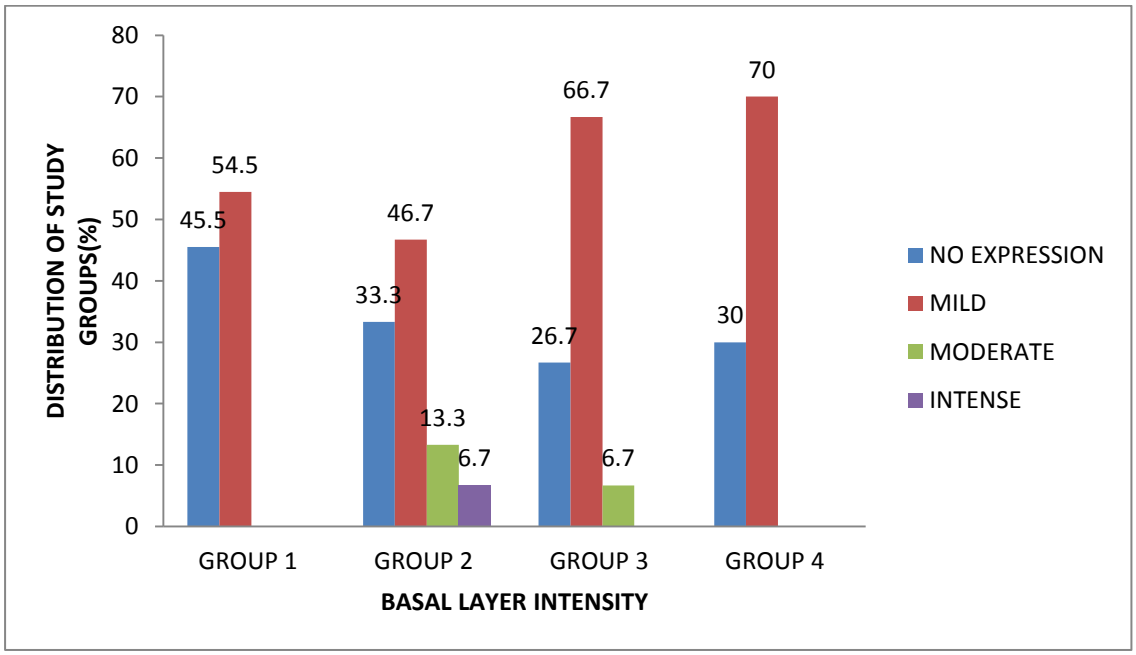
GROUP IV – Normal mucosa (n=10)

TABLE 8: COMPARISON OF BASAL LAYER INTENSITY

BETWEEN THE STUDY GROUPS (N=51)

BASAL LAYER INTENSITY	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	5(45.5%)	5(33.3%)	4(26.7%)	3(30%)	0.682
MILD	6(54.5%)	7(46.7%)	10(66.7%)	7(70%)	
MODERATE	0(0%)	2(13.3%)	1(6.7%)	0(0%)	
INTENSE	0(0%)	1(6.7%)	0(0%)	0(0%)	

GRAPH 8: COMPARISON OF BASAL LAYER INTENSITY AMONG THE STUDY GROUPS



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

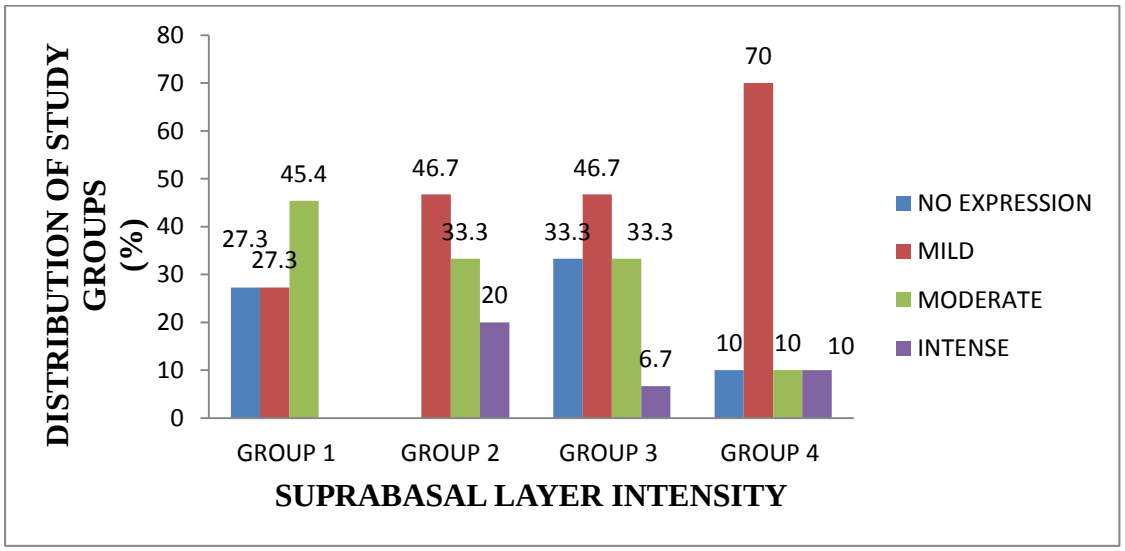
GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 9: COMPARISON OF SUPRABASAL LAYER INTENSITY BETWEEN THE STUDY GROUPS (N=51)

SUPRABASAL LAYER INTENSITY	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	3(27.3%)	0(0%)	2(33.3%)	1(10%)	0.268
MILD	3(27.3%)	7(46.7%)	7(46.7%)	7(70%)	
MODERATE	5(45.4%)	5(33.3%)	5(33.3%)	1(10%)	
INTENSE	0(0%)	3(20%)	1(6.7%)	1(10%)	

GRAPH 9: COMPARISON OF SUPRA BASAL LAYER INTENSITY BETWEEN THE STUDY GROUPS (N=51)



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

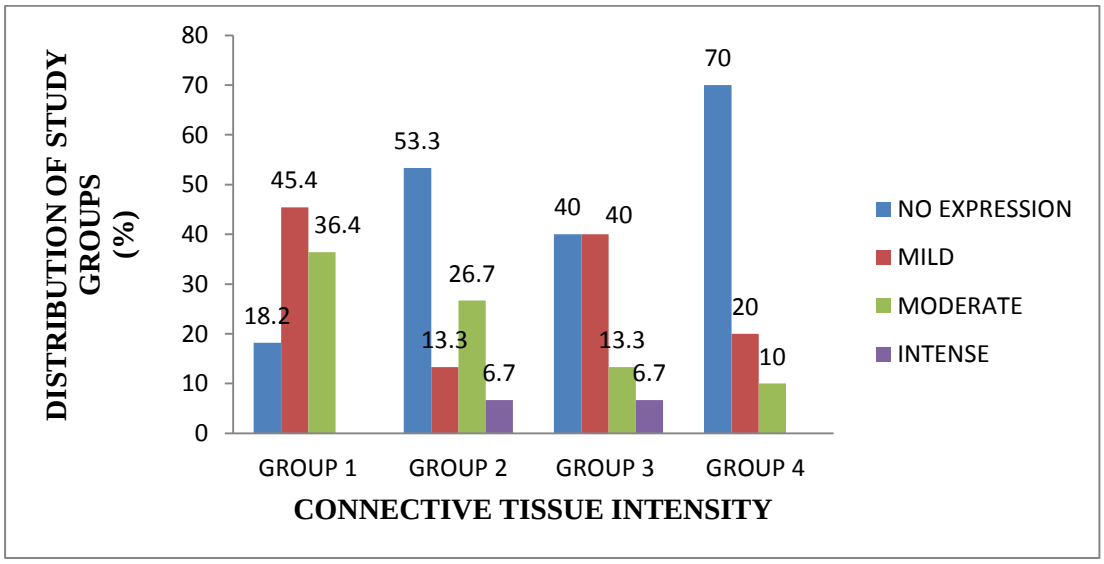
GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 10: COMPARISON OF CONNECTIVE TISSUE INTENSITY BETWEEN THE STUDY GROUPS (N=51)

CONNECTIVE TISSUE INTENSITY	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	2(18.2%)	8(53.3%)	6(40%)	7(70%)	0.319
MILD	5(45.4%)	2(13.3%)	6(40%)	2(20%)	
MODERATE	4(36.4%)	4(26.7%)	2(13.3%)	1(10%)	
INTENSE	0(0%)	1(6.7%)	1(6.7%)	0(0%)	

GRAPH 10: COMPARISON OF CONNECTIVE TISSUE INTENSITY BETWEEN THE STUDY GROUPS



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

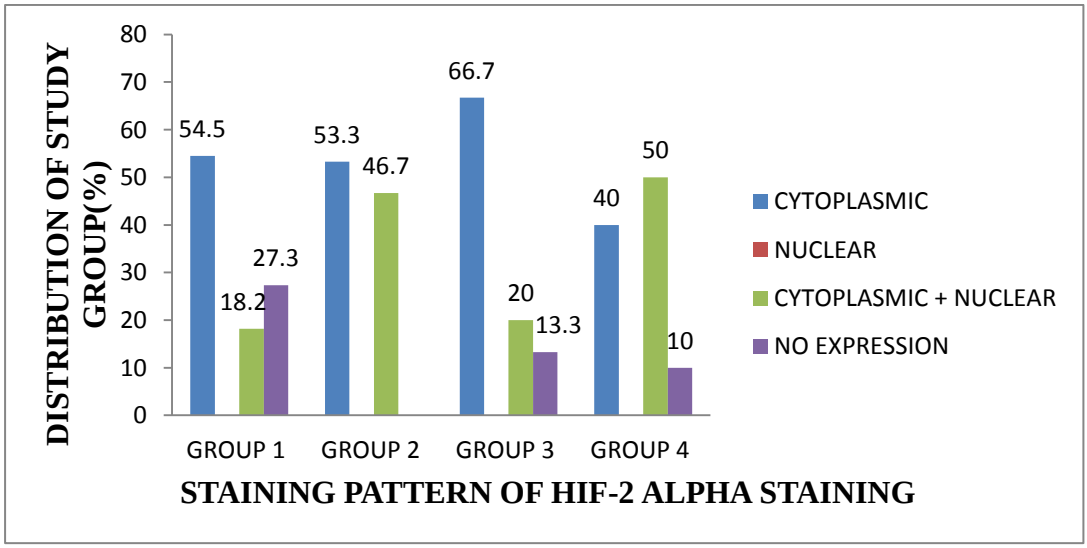
GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 11: DISTRIBUTION OF STAINING PATTERN OF HIF-2 ALPHA AMONG THE STUDY GROUPS (N=51)

STAINING PATTERN	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
CYTOPLASMIC	6(54.5%)	8(53.3%)	10(66.7%)	4(40%)	0.234
NUCLEAR	0(0%)	0(0%)	0(0%)	0(0%)	
CYTOPLASMIC + NUCLEAR	2(18.2%)	7(46.7%)	3(20%)	5(50%)	
NO EXPRESSION	3(27.3%)	0(0%)	2(13.3%)	1(10%)	

GRAPH 11: DISTRIBUTION OF STAINING PATTERN OF HIF-2 ALPHA AMONG THE STUDY GROUPS (N=51)



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

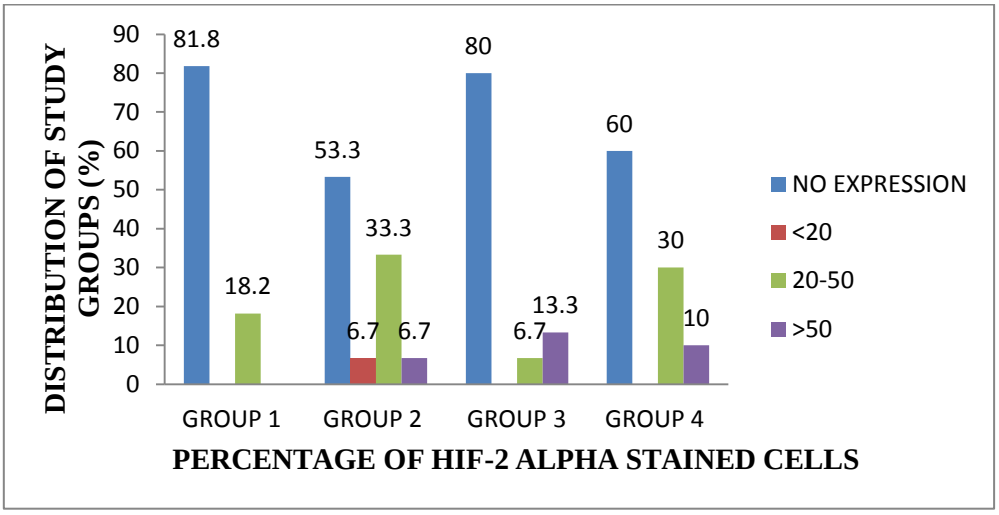
GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 12: NUCLEAR LABELLING INDEX OF HIF-2 ALPHA IN THE STUDY GROUPS (N=51)

PERCENTAGE OF STAINED CELLS	GROUP 1 (n=11)	GROUP 2 (n=15)	GROUP 3 (n=15)	GROUP 4 (n=10)	p VALUE
NO EXPRESSION	9(81.8%)	8(53.3%)	12(80%)	6(60%)	0.534
<20	0(0%)	1(6.7%)	0(0%)	0(0%)	
20-50	2(18.2%)	5(33.3%)	1(6.7%)	3(30%)	
>50	0(0%)	1(6.7%)	2(13.3%)	1(10%)	

GRAPH 12: NUCLEAR LABELLING INDEX OF HIF-2 ALPHA IN THE STUDY GROUPS



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 13: COMPARISON OF HIF-2 ALPHA MEAN LABELLING INDEX BETWEEN THE STUDY GROUPS

GROUPS	N	MEAN ± STANDARD DEVIATION	OVERALL p-VALUE
GROUP 1 Vs 2	2	10.975 ± 19.21	0.282
	7	18.49 ± 22.88	
GROUP 1 Vs 3	2	10.975 ± 19.21	
	3	7.85 ± 18.08	
GROUP 1 Vs 4	2	10.975 ± 19.21	
	4	12.24 ± 24.08	
GROUP 2 Vs 3	7	18.49 ± 22.88	
	3	7.85 ± 18.08	
GROUP 2 Vs 4	7	18.49 ± 22.88	
	4	12.24 ± 24.08	
GROUP 3 Vs 4	3	7.85 ± 18.08	
	4	12.24 ± 24.08	

GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

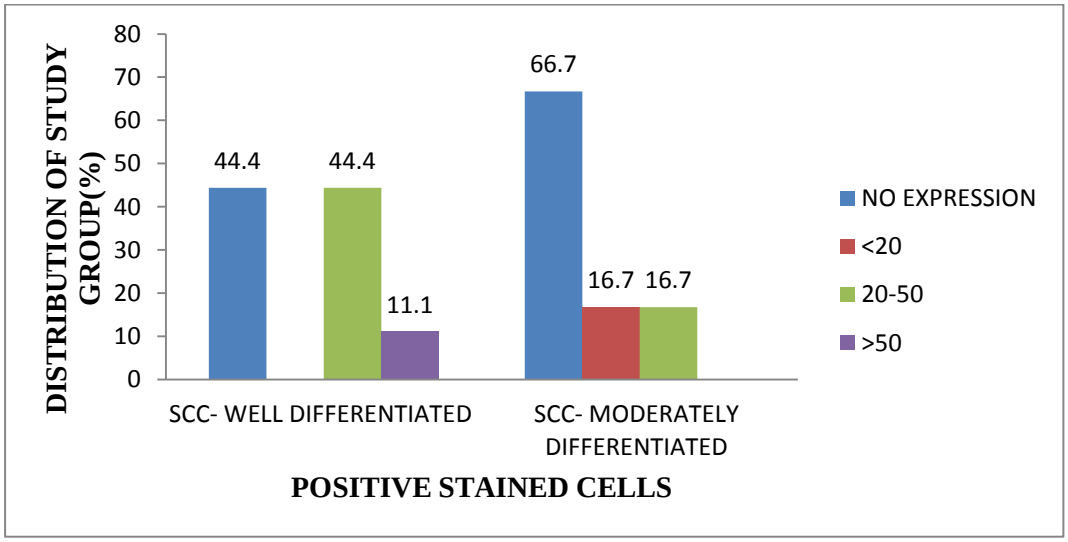
GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 14: DISTRIBUTION OF HIF-2 ALPHA POSITIVE STAINED CELLS IN GRADES OF OSCC (GROUP 2) (N = 15)

PERCENTAGE OF STAINED CELLS	SCC - WELL DIFFERENTIATED (n=9)	SCC - MODERATELY DIFFERENTIATED (n=6)	p VALUE
NO EXPRESSION	4(44.4%)	4(66.7%)	0.343
<20	0(0%)	1(16.7%)	
20-50	4(44.4%)	1(16.7%)	
>50	1(11.1%)	0(0%)	

GRAPH 14: DISTRIBUTION OF HIF-2 ALPHA POSITIVE STAINED CELLS IN GRADES OF OSCC (GROUP 2) (N = 15)



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

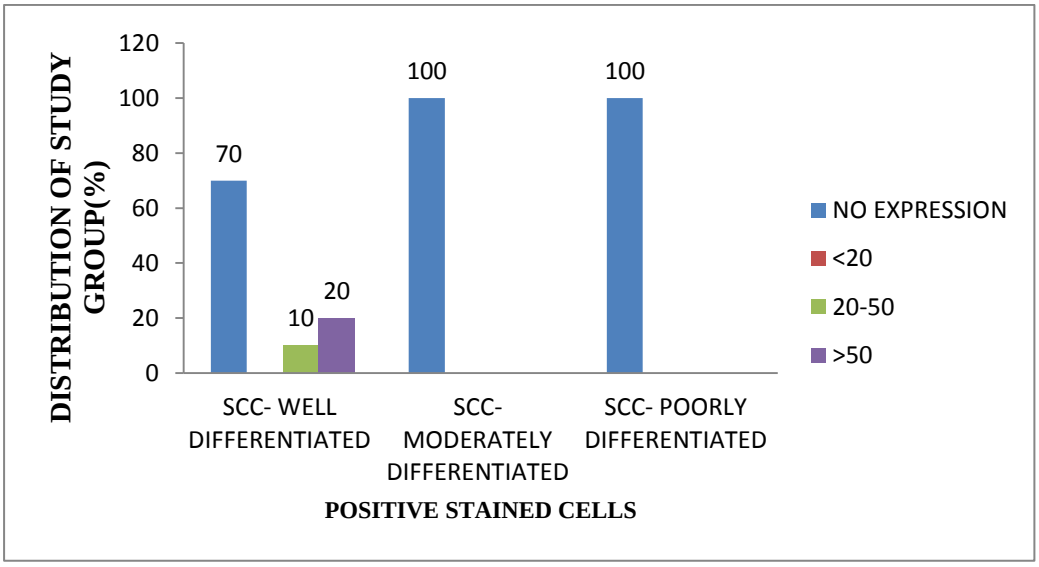
GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 15: DISTRIBUTION OF HIF-2 ALPHA POSITIVE STAINED CELLS IN GRADES OF OSCC (GROUP 3) (N = 15)

PERCENTAGE OF STAINED CELLS	SCC - WELL DIFFERENTIATED (n=10)	SCC - MODERATELY DIFFERENTIATED (n=2)	SCC- POORLY DIFFERENTIATED (n=3)	p VALUE
NO EXPRESSION	7(70%)	2(100%)	3(100%)	0.759
<20	0(0%)	0(0%)	0(0%)	
20-50	1(10%)	0(0%)	0(0%)	
>50	2(20%)	0(0%)	0(0%)	

GRAPH 15: DISTRIBUTION OF HIF-2 ALPHA POSITIVE STAINED CELLS IN GRADES OF OSCC (GROUP 3) (N = 15)



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

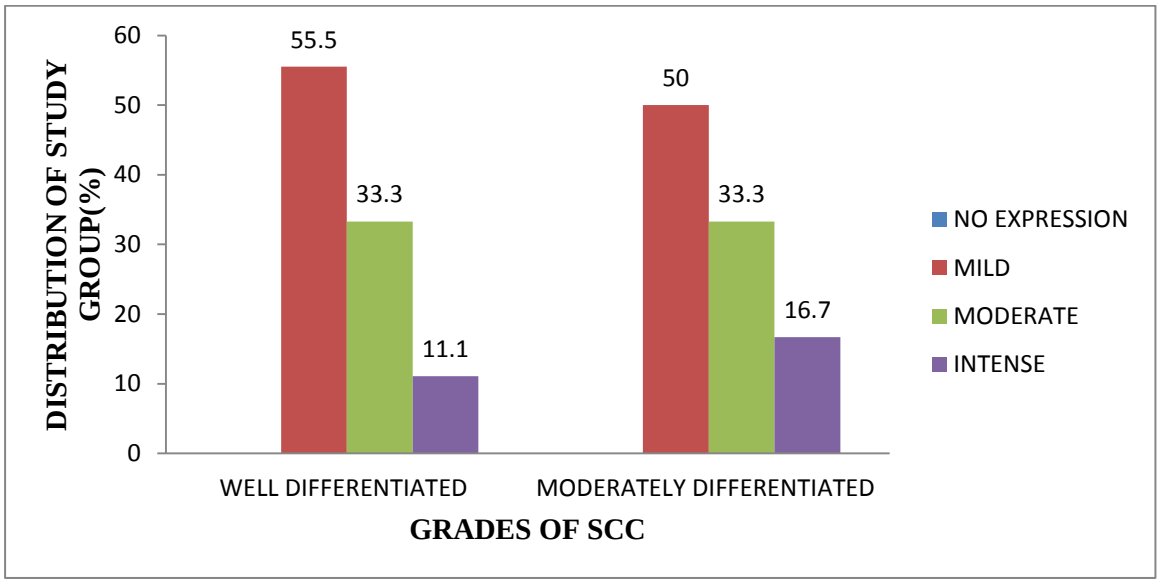
TABLE 16: COMPARISON OF INTENSITY OF HIF-2 ALPHA

BETWEEN GRADES OF OSCC (GROUP 2) N=15

GRADES OF SCC	NO EXPRESSION	MILD	MODERATE	INTENSE	p VALUE
WELL DIFFERENTIATED (n=9)	0(0%)	5(55.5%)	3(33.3%)	1(11.1%)	0.949
MODERATELY DIFFERENTIATED (n=6)	0(0%)	3(50%)	2(33.3%)	1(16.7%)	

GRAPH 16: COMPARISON OF INTENSITY OF HIF-2 ALPHA

BETWEEN GRADES OF OSCC (GROUP 2) N=15



GROUP I – Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

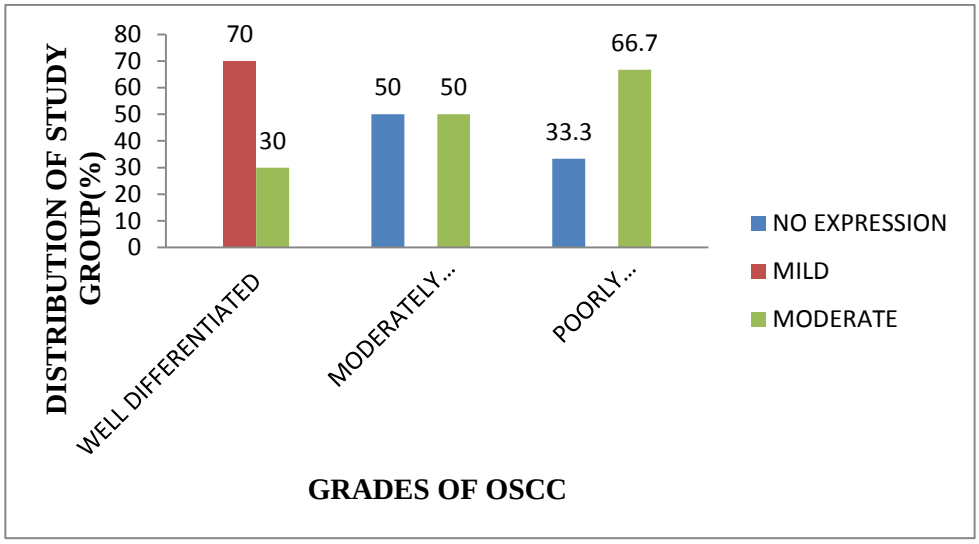
GROUP III – Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 17: COMPARISON OF INTENSITY OF HIF-2 ALPHA BETWEEN GRADES OF OSCC (GROUP 3) N=15

GRADES OF SCC	NOT EXPRESSED	MILD	MODERATE	p VALUE
WELL DIFFERENTIATED (n=10)	0(0%)	7(70%)	3(30%)	0.072
MODERATELY DIFFERENTIATED (n=2)	1(50%)	0(0%)	1(50%)	
POORLY DIFFERENTIATED (n=3)	1(33.3%)	0(0%)	2(66.7%)	

GRAPH 17: COMPARISON OF INTENSITY OF HIF-2 ALPHA BETWEEN GRADES OF OSCC (GROUP 3) N=15



GROUP I –Oral Submucous Fibrosis (n=11)

GROUP II – Oral Squamous cell carcinoma with areca nut usage (n=15)

GROUP III –Oral Squamous cell carcinoma without areca nut usage (n=15)

GROUP IV – Normal mucosa (n=10)

TABLE 18: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 1 AND GROUP 2

GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 1 (n=11)	0(0%)	5(45.4%)	6(54.5%)	0(0%)	0.329
GROUP 2 (n=15)	0(0%)	8(54%)	5(33.3%)	2(13.3%)	

TABLE 19: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 1 AND GROUP 3

GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 1 (n=11)	0(0%)	5(45.4%)	6(54.5%)	0(0%)	0.415
GROUP 3 (n=15)	2(13.3%)	7(46.7%)	6(40%)	0(0%)	

TABLE 20: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 1 AND GROUP 4

GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 1 (n=11)	0(0%)	5(45.4%)	6(54.5%)	0(0%)	0.359
GROUP 4 (n=10)	1(10%)	6(60%)	3(30%)	0(0%)	

TABLE 21: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 2 AND GROUP 3

GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 2 (n=15)	0(0%)	8(54%)	5(33.3%)	2(13.3%)	0.245
GROUP 3 (n=15)	2(13.3%)	7(46.7%)	6(40%)	0(0%)	

TABLE 22: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 2 AND GROUP 4

GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 2 (n=15)	0(0%)	8(54%)	5(33.3%)	2(13.3%)	0.407
GROUP 4 (n=10)	1(10%)	6(60%)	3(30%)	0(0%)	

TABLE 23: INTERCOMPARISON OF INTENSITY BETWEEN GROUP 3 AND GROUP 4

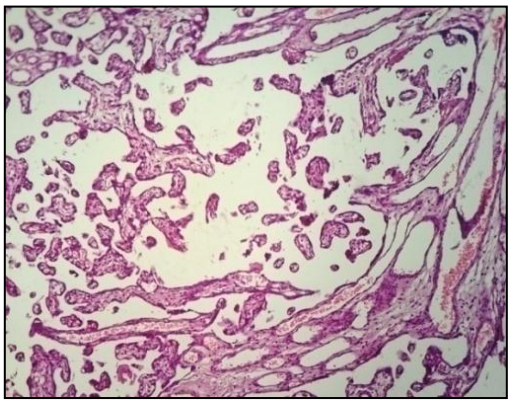
GROUP	NOT EXPRESSED	MILD	MODERATE	INTENSE	p VALUE
GROUP 3 (n=15)	2(13.3%)	7(46.7%)	6(40%)	0(0%)	0.808
GROUP 4 (n=10)	1(10%)	6(60%)	3(30%)	0(0%)	

Photographs

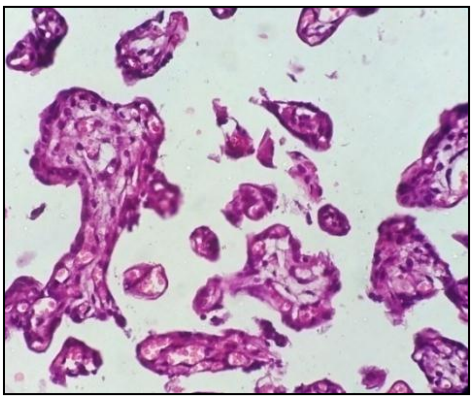
HIF-2 ALPHA POSITIVE CONTROL

HEMATOXYLIN & EOSIN STAIN

10X

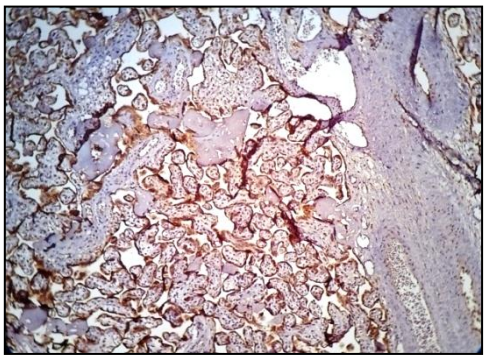


40X

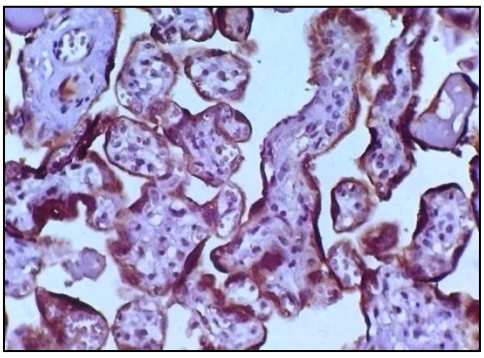


HIF-2 ALPHA STAIN

10X



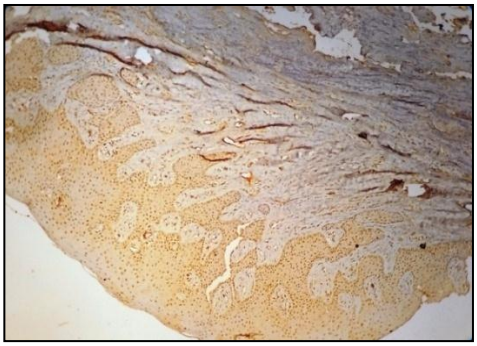
40X



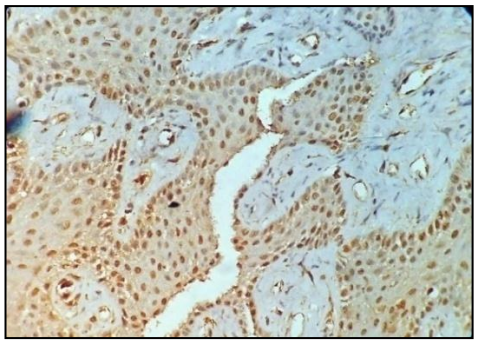
HIF-2 ALPHA EXPRESSION IN NORMAL MUCOSA

`

HIF-2α – 10X

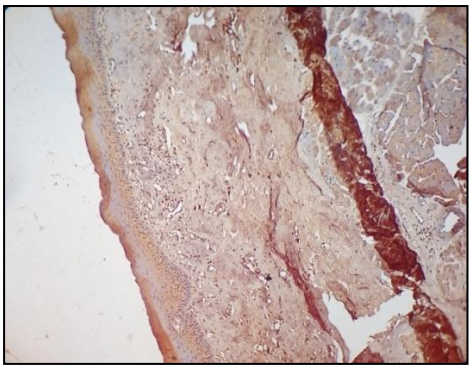


HIF-2α – 40X

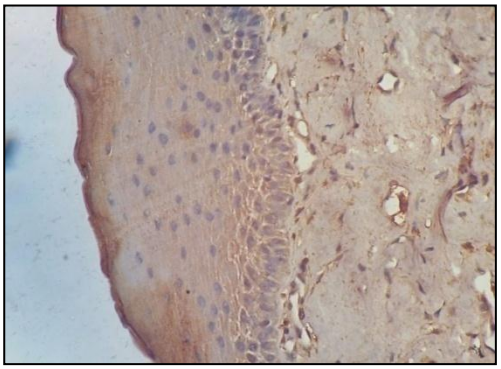


HIF-2 ALPHA EXPRESSION IN ORAL SUBMUCOUS FIBROSIS

HIF-2α – 10X

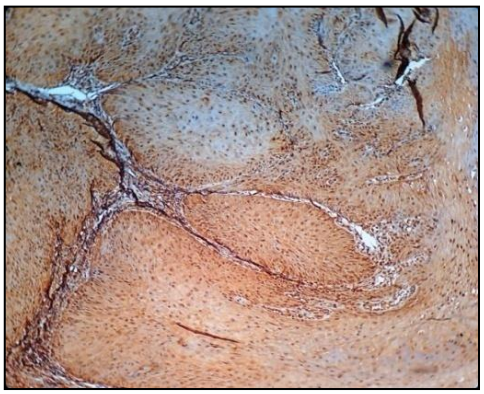


HIF-2α – 40X

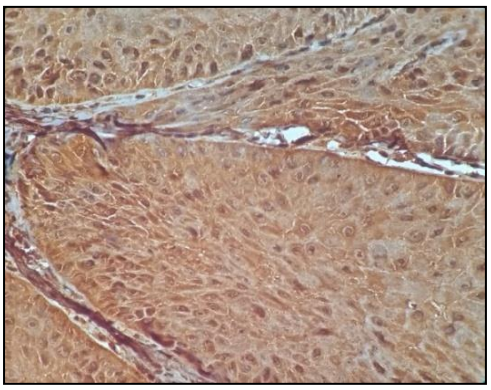


HIF-2 ALPHA EXPRESSION IN OSCC WITH ARECA NUT HABIT

HIF-2α – 10X

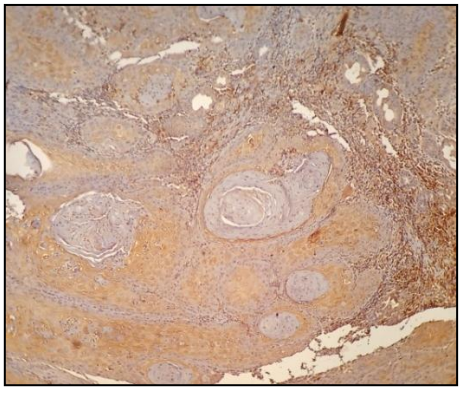


HIF-2α – 40X

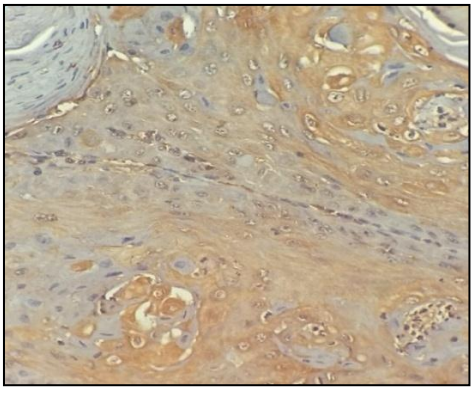


HIF-2 ALPHA EXPRESSION IN OSCC WITHOUT ARECA NUT HABIT

HIF-2α – 10X

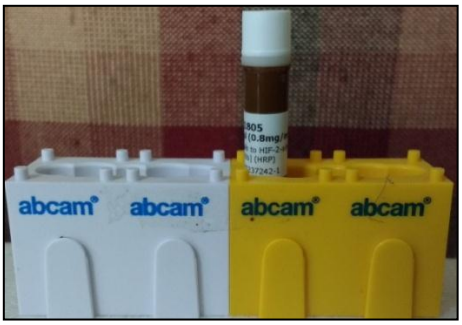


HIF-2α – 40X



ANTIBODY KIT

PRIMARY ANTIBODY (HIF-2 ALPHA)



SECONDARY ANTIBODY

ARMAMENTARIUM

Discussion

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of oral malignancies and is the 11th most common cancer worldwide; it provides 3% of all newly diagnosed cancer cases [22]. Clinical premalignant lesions and conditions like oral leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF) precede the transformation to squamous cell carcinoma.

Among these, OSMF, a chronic fibrotic premalignant condition, demonstrates a regional distribution, being particularly prevalent in the Indian subcontinent. Scientific literature to date provides firm evidence that areca nut is the major etiological factor for this disease but the exact mechanism of action on the oral tissues remains to be elucidated [24].

The disease is characterized by inflammation and progressive generalized submucosal fibrosis, leading to limitation of mouth opening. It exhibits characteristic histopathological features that include juxtaepithelial hyalinization and excessive collagen deposition in the connective tissue, secondary to which the epithelium becomes atrophic. This atrophic epithelium is prone to injury by the areca nut extracts that predispose to the development of malignancy [13,24].

The most widespread form of tobacco habit is chewing of betel-quid with tobacco and this has been demonstrated as a major risk factor for cancer of oral cavity. Betel quid with or without tobacco is one of the independent major risk factors for oral cancer. Apart from tobacco use, ill-fitting dentures, poor oral hygiene, syphilis, inadequate diet, malnutrition and chronic irritation

from rough or broken teeth are reported more frequently in oral cancer patients[47].

OSMF is characterized by both fibrosis and malignant transformation in the background of fibrosis. Hypoxia has been reported to be associated with fibrosis in other organs of the body as it has been shown that renal fibroblasts reduce their turnover and upregulate matrix production when exposed to hypoxia. It has also been reported that lung fibroblasts produce augmented levels of TGF-β and enhanced accumulation of collagen during hypoxia. Hypoxia-inducible factors are heterodimeric transcription factors with α and β subunits. HIFs regulate the expression of genes controlling glucose uptake, metabolism, angiogenesis, erythropoiesis, cell proliferation and apoptosis. HIF is mainly activated under hypoxic conditions [48]. In addition, molecules which are upregulated by hypoxia such as hypoxia inducible factor-1alpha (HIF-1α) are said to be associated with malignant transformation of epithelium in other sites [3].

Hypoxia inducible factors (HIF)-1α and HIF-2α are nuclear transcription factors that regulate the cellular response to hypoxia and are important for solid tumor growth and survival and promotion of aggressiveness leading to poor prognosis [49].

Given the fact that overexpression of HIF-2α has poor prognosis and less survival in many epithelial cancers, this study was done in oral submucous fibrosis and OSCC using immunohistochemistry to study the

expression of HIF-2α comparing with that of the normal mucosa and also ascertain its role in malignant transformation of OSMF.

Archival blocks were used in the study groups. There was a significant difference with respect to age in our study between the groups. We observed that 37% of group 1 (OSMF) and 40% of group 2 (OSCC with areca nut habit) were in the age group of 20-40 years, whereas, only 6% of those with group 3 (OSCC without areca nut habit) were observed in this age group. This indicates that patients with areca nut chewing habit, were in the younger age group as seen in group 1 (OSMF). In our study, we also observed that in group 2 (OSCC with areca nut habit), our cases were in the younger age group and this could be attributed to the habit of chewing areca nut, which is prevalent among young people compared to group 3 (OSCC without areca nut habit) patients who were in the older age group. This finding was similar to the study by **Chaturvedi P *et al***, where, out of the 371 patients who had OSCC, 112 patients had OSCC with OSMF and they belonged to the younger age group[50]. Likewise, **Balaji P *et al*** reported a male patient of age 32 years who was diagnosed to have well differentiated OSCC with OSMF. Therefore, it is evident that areca nut promotes cancer in younger age group[51].

94% of the patients in group 3 (OSCC without areca nut habit) were above 40 years. This pattern of age distribution for OSCC was consistent with that of **Massarelli E *et al*** in whose study 89% of OSCC cases were seen in the age group above 40 years [52].

In our study the gender distribution among the study groups showed a male predominance. In all the three groups (OSMF, OSCC with areca and OSCC without areca), males were predominantly more affected than females. This pattern of gender distribution was consistent with **Lee CH *et al*** in whose study 98% of OSMF were males [53]. **Chen YJ *et al*** in their study of 703 OSCC cases, reported that 93% of the study population were males. Thus the author suggests that the prevalence of OSCC was common in males compared to females [54].

In group 1 (OSMF), 82% of patients had the habit of chewing areca nut either alone or in combination with tobacco and alcohol which is consistent with the finding that chewing areca nut is the main etiological factor for the development of OSMF and has a synergistic role when used along with tobacco or alcohol in promoting cancer [55]. In group 2 (OSCC with areca nut habit), all the patients had the habit of chewing areca nut either alone or in combination with tobacco and alcohol. Though, we could not elicit the confirmed histopathological diagnosis of OSMF in all the patients in group 2 (OSCC with areca nut habit), 4 patients had history of difficulty in opening the mouth and the presence of fibrous bands. In these patients, we believe that there was a malignant transformation of pre-existing OSMF.

In our study, 87% of group 3 (OSCC without areca nut habit) patients had no habits and 13% of patients used tobacco either by smokeless or chewable form or by smoking. The absence of habits and the occurrence of

oral cancer is in concurrence with the study by **Cruz I *et al* (2002)** in which a subgroup of patients were identified, in whom no known carcinogenic factors (habits) could be found and **Ho PS *et al***, who in their prospective study of 148 cases with OSCC, found that 40% did not have any habits, owing to alternative mechanisms of oral carcinogenesis [56,57]. In our study, in group 3 (OSCC without areca nut habit) in patients who did not have habits, the pathogenesis of OSCC could have been due to aberrant DNA methylation, loss of chromosomal heterozygosity, telomerase expression and various oncogenes.

In this study, buccal mucosa was the most common site of biopsy in all cases of group 1 (OSMF) and 46% of group 2 (OSCC with history of using areca nut). This finding was similar to that of **Ho PS *et al*** who stated in their cohort of 148 cases, that buccal mucosa was the common site of occurrence for OSMF and OSCC [57]. Interestingly, in group 2 (OSCC with areca nut habit), 40% of cases who used areca alone or used areca along with smoking, presented with OSCC in the lateral border of tongue.

In our study, in group 3 (OSCC without areca nut habit), 87% of cases did not have any habit and all of them presented with OSCC in the lateral border of tongue. There were 2 patients in this group who had habits. One had the habit of pouching tobacco and the other had the habit of only smoking tobacco and the site of occurrence was buccal vestibule and anterior palate respectively in these 2 patients. This finding was consistent with **Chen YJ *et***

al in whose study nearly 35% of OSCC occurred on lateral border of tongue and none of their patients had habits [54].

In our study, all the cases from group 1 (OSMF) and group 2 (OSCC with areca nut habit) had positive expression of HIF-2α whereas 87% of cases from group 3 (OSCC without areca nut habit) and 90% of cases of group 4 (normal mucosa) did not express HIF-2α.

In our study, all the cases (100%) from group 1 (OSMF) and 2 (OSCC with areca nut habit), expressed HIF-2α. 53% of cases in group 1 (OSMF), 46% of cases in group 2 (OSCC with areca nut habit) and 52% of cases in group 3 (OSCC without areca nut habit) had expression in the basal, suprabasal layers of epithelium as well as the connective tissue. In group 2 (OSCC with areca nut habit), basal and suprabasal expression was seen in 20% of cases and only suprabasal expression was seen in 33% of the cases. This finding indicates that areca nut, could influence the differentiation of epithelial cells due to hypoxic stress as stated by **Pahlman S *et al*** [45]. The pattern of tissue localisation, in other areas of the epithelium was almost similar.

There was significant difference in expression of HIF-2α in the connective tissue between the groups. Its expression was seen only in group 1 (OSMF). This can be explained by the finding by **Fraisl P *et al,*** who reported that hypoxic stress activates HIF-2α 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 [58]. Due to extensive fibrosis and hyalinisation in OSMF, there could be alterations in the vasculature and predominant expression of HIF-2α in the connective tissue stroma of OSMF.

There was a significant difference in the proportion of cases, with respect to the expression in suprabasal layer and basal & suprabasal layer between the groups. This finding of significant expression in cases of group 1 (OSMF) and group 2 (OSCC with areca nut habit) in the basal and suprabasal layers highlights the role of hypoxia in transformation of OSMF to OSCC and this could be due to the influence of areca nut [4].

Comparing the study groups, all the cases from group 1 (OSMF) and group 2 (OSCC with areca nut habit) had expressed HIF-2α whereas, 13% of cases from group 3 (OSCC without areca nut habit) and 10% of cases from the control group did not express. Most of the cases among the study groups which expressed HIF-2α showed mild to moderate intensity. Intense expression was seen only in group 2 (OSCC with areca nut habit) in 13% of cases. This intense intensity of staining in areca nut users of group 2 (OSCC with areca nut habit) was concurrent with that of **Koukourakis MI *et al***, who studied specific hypoxia targets or antiangiogenic agents in order to predict the causes of therapeutic failure of OSCC after radiation. They stated that CAIX (Carbonic anhydrase 9), is a hypoxia inducible transmembrane enzyme and has been shown to correlate with direct measurement of oxygen tension in

cervical cancer. In their study they have also shown that the 2 factors regulate different pathways of hypoxia viz., CAIX and HIF-2α and stated that hypoxia inducible CAIX is regulated by HIF-1 and HIF-2α. In their study, they concluded that significant association of high HIF-2α and CAIX reactivity with poor locoregional control prevailed in head & neck SCCs. In our study based on the finding of intense expression in group 2 (OSCC cases who used areca nut), we hypothesise that areca nut could have assisted in the pathogenesis of OSCC in association with CAIX which is also found in areca nut chewers [59].

In the basal layer, all the study groups showed predominantly mild expression of HIF-2α, of which, 20% of cases in group 2 (OSCC with areca nut habit) had moderate to intense intensity & 7% of cases in group 3 (OSCC without areca nut habit) had moderate expression in the basal layer.

45% of cases in group 1 (OSMF), 52% of cases in group 2 (OSCC with areca nut habit) and 40% of cases in group 3 (OSCC without areca nut habit) showed moderate to intense expression of HIF-2α. The maximum number of cases which expressed in connective tissue were in group 1 (OSMF). There was no significant difference in the intensity of staining between the groups.

This observation from our study was also in concurrence with the finding by **Pahlman S *et al***, who stated that HIF-2α can accumulate and get activated in response to hypoxic stress. This was explained by their

immunohistochemical study of the oxygen sensitive HIF-alpha subunits using tumors from a transgenic mouse mammary tumor model injected with Hypoxyprobe as reference tissue. The mammary gland of mouse was studied during the development, lactation and involution stages. Since hypoxia affects normal epithelial differentiation, HIFs were considered important for normal breast epithelial development and regeneration as well as cancer initiation and progression. It has been shown in breast epithelium that HIF-2α is associated with breast cancer metastasis and poor patient survival. It has also been reported that hypoxia and the transcriptional activity are linked to a state of loss of polarisation and a cancer like phenotype in primary human breast epithelial cells [45].

Based on the findings in the breast epithelial cells, we extend the analogy, to oral epithelium and postulate that the significant intense expression of HIF-2α, in OSMF and OSCC which was associated to areca nut, as a marker of malignant transformation of OSMF in the presence of the habit of chewing areca nut.

When we studied nuclear and cytoplasmic staining pattern, we observed that though nearly 49% of cases in group 2 (OSCC with areca nut habit) showed nuclear and cytoplasmic staining which was highest among the groups, there was no significant difference in the pattern of staining between the groups. In their study, **Pahlman S *et al*** reported that HIF-2α 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 indicates that under normoxic condition, HIF-2α is regulated by prolyl hydroxylase-domain enzymes (PHDs) that initiates its degradation through the von Hippel-Lindau protein (tumor suppressor protein) but in hypoxic conditions, the activity of prolyl hydroxylase-domain enzymes is inhibited and HIF-2α is not degraded but enters the nucleus and binds to a conserved DNA sequence (hypoxia responsive element) and initiates the transactivation of hypoxia-responsive genes. Therefore, HIF-2α plays a role in the hypoxic regulation of translation by binding to mRNA in the cytoplasm [45].

In our study, when we compared the intensity of staining between the grades of OSCC in group 2 (OSCC with areca nut habit) and group 3 (OSCC without areca nut habit), we observed an increase of staining, though not significant, from well differentiated to moderately differentiated and poorly differentiated carcinoma. This was concurrent with the finding of **Raval RR *et al***, in whose study the expression of HIF-2α increased with the degree of dysplasia in preneoplastic kidney lesions, and therefore it has a role in the transformation of dysplastic cells [6,60].

The nuclear labelling index was also ascertained based on the percentage of stained cells but the difference among the groups was not significant.

We also studied the number of positive nuclei in groups 2 and 3 in relation to tumor differentiation. In group 2 (OSCC with areca nut habit),

55% of nuclei stained positively in well differentiated OSCC and 33% of positive nuclei were observed in the moderately differentiated OSCC. In group 3 (OSCC without areca nut habit), well differentiated OSCC had 30% of nuclei stained positive whereas moderately and poorly differentiated OSCC had 100% negative expression in nuclei.

Our findings show that, 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 the expression of HIF-2α. This was consistent with the findings of **Talks KL *et al,*** who stated that 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 [7].

In the present study, the mean labelling index of HIF-2α in group 2 (OSCC with areca nut habit) was found to be non-significantly higher than that of group 3 (OSCC without areca nut habit) and group 1 (OSMF).

Summary and Conclusion

- A total of 51 patients were included in this study, comprising of 11 cases of group 1 (OSMF), 15 cases of group 2 (OSCC with areca nut habit), 15 cases of group 3 (OSCC without areca nut habit) and 10 cases of group 4 (normal mucosa).
- In group 1 (OSMF), group 2 (OSCC with areca nut habit) and in group 3 (OSCC without areca nut habit), 54%, 47% and 67% of cases were in the age group of 41-60 years respectively. 40% of cases in group 2 (OSCC with areca nut habit) were in the age group of 20-40 years. This age distribution was statistically significant. (p=0.006)
- Out of 51 cases, 77% were males and 23% were females among the study groups.
- In our study, 82% of cases in group 1 (OSMF) and 100% of cases in group 2 (OSCC with areca nut habit) either used areca nut alone or in combination with tobacco and alcohol. In group 3 (OSCC without areca nut habit), 87% of cases had no habits.
- In group 1 (OSMF) and group 2 (OSCC with areca nut habit), the most common site of biopsy was the buccal mucosa. 47% of cases had the lateral border of tongue as the most common site of biopsy in group 3 (OSCC without areca nut habit).
- All the study samples expressed HIF-2α in Group 1 (OSMF) and group 2 (OSCC with areca nut habit) except 13% and 10% of cases in group

3 (OSCC without areca nut habit) and group 4 (normal mucosa) respectively.

- Tissue localisation of the HIF-2α expression was present in basal, suprabasal layers of epithelium and connective tissue. 27% of cases in group 1 (OSMF) had expression in connective tissue alone and 33% of cases in group 2 (OSCC with areca nut habit) had expression in the suprabasal layer alone. All the study groups showed either cytoplasmic or both cytoplasmic and nuclear expression.
- There was a significant difference in the proportion of cases, with respect to the expression in suprabasal layer and basal & suprabasal layer between the groups.
- In the basal layer, all of the study groups had mild expression, with 13% and 7% of cases in group 2 (OSCC with areca nut habit) which had moderate and intense expression of HIF-2α respectively.
- In the suprabasal layer, 33% of cases in group 2 (OSCC with areca nut habit) and group 3 (OSCC without areca nut habit) had moderate intensity respectively and 20% of cases in group 2 (OSCC with areca nut habit) had intense expression. In group 1 (OSMF), 45% of cases had moderate intensity of HIF-2α.
- In the connective tissue, there was no significant difference in HIF-2α expression among the study groups.

- There was significant intense expression of HIF-2α, in OSMF and OSCC associated with areca nut.
- The mean labelling index of HIF-2α in group 2 - OSCC with areca nut habit (18.49) > group 4 - normal (12.24) > group 1 - OSMF (10.975) > group 3 - OSCC without areca nut habit (7.85) [p value = 0.282]
- The number of positive stained nuclei decreased as the tumor progressed from well to poor differentiation in both group 2(OSCC with areca nut habit) and group 3 (OSCC without areca nut habit) cases.
- There was an increase in HIF-2α expression as the tumor progressed from well to poor differentiation in group 2 (OSCC with areca nut habit) and group 3 (OSCC without areca nut habit) cases.

CONCLUSION:

In our study, based on the pattern and intensity of HIF-2 alpha expression in oral submucous fibrosis and oral squamous cell carcinoma with areca nut habit, we hypothesize that altered HIF-2 alpha expression, is due to the effect of areca nut on tissues, and could be an indication for malignant transformation.

Bibliography

1. **Wang B, Zhang S, Yue K, Wang XD.**

   The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases.

   Chinese Journal of Cancer. 2013;32(11):614.

2. **Kumar K, Saraswathi TR, Ranganathan K, Devi U, Elizabeth J.**

   Oral submucous fibrosis: A clinico-histopathological study in Chennai.

   Indian Journal of Dental Research. 2007;18(3):106.

3. **Tilakaratne WM, Iqbal Z, Teh MT, Ariyawardana A, Pitiyage G, Cruchley A, Stewart JE, Hagi-Pavli E, Lalli A, Waseem A, Parkinson EK.**

   Upregulation of HIF-1α in malignant transformation of oral submucous fibrosis.

   Journal of Oral Pathology & Medicine. 2008;37(6):372-7.

4. **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: JOMFP. 2015 May;19(2):122.

5. **Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC.**

   Differential roles of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in hypoxic gene regulation.

Molecular and cellular biology. 2003 Dec 15;23(24):9361-74.

6. **Zhao J, Du F, Shen G, Zheng F, Xu B.**

   The role of hypoxia-inducible factor-2 in digestive system cancers.

   Cell death & disease. 2015 Jan 1;6(1):e1600.

7. **Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL.**

   The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages.

   The American journal of pathology. 2000 Aug 31;157(2):411-21.

8. **Liu W, Wang SJ, Lin QD.**

   Study on the Expressions of PHD and HIF in Placentas from Normal Pregnant Women and Patients with Preeclampsia.

   International journal of biological sciences. 2014;10(3):278.

9. **Rajakumar A, Whitelock KA, Weissfeld LA, Daftary AR, Markovic N, Conrad KP.**

   Selective overexpression of the hypoxia-inducible transcription factor, HIF-2α, in placentas from women with preeclampsia.

   Biology of Reproduction. 2001; 64(2): 499-506.

10. **Tilakaratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S.**

    Oral submucous fibrosis: review on aetiology and pathogenesis.

    Oral oncology. 2006 Jul 31;42(6):561-8.

11. **Trivedy CR, Warnakulasuriya KA, Peters TJ, Senkus R, Hazarey VK, Johnson NW.**

    Raised tissue copper levels in oral submucous fibrosis.

    Journal of oral pathology & medicine. 2000;29(6):241-8.

12. **Sharan RN, Mehrotra R, Choudhury Y, Asotra K.**

    Association of betel nut with carcinogenesis: revisit with a clinical perspective.

    PloS one. 2012;7(8):e42759.

13. **Prabhu RV, Prabhu V, Chatra L, Shenai P, Suvarna N, Dandekeri S.**

    Areca nut and its role in oral submucous fibrosis.

    Journal of clinical and experimental dentistry. 2014;6(5):e569.

14. **Ahmad MS, Ali SA, Ali AS, Chaubey KK.**

    Epidemiological and etiological study of oral submucous fibrosis among gutkha chewers of Patna, Bihar, India.

    Journal of Indian society of Pedodontics and Preventive dentistry. 2006;24(2):84.

15. **Namboodiripad PA**.

    Cystatin C: Its role in pathogenesis of OSMF.

    Journal of Oral Biology and Craniofacial Research. 2014;4(1):42-6.

16. Shieh DH, Chiang LC, Shieh TY.

Augmented mRNA expression of tissue inhibitor of metalloproteinase-1 in buccal mucosal fibroblasts by arecoline and safrole as a possible pathogenesis for oral submucous fibrosis.

Oral oncology. 2003;39(7):728-35.

17. Chang YC, Yang SF, Tai KW, Chou MY, Hsieh YS.

Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinaseA activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis.

Oral oncology. 2002;38(2):195-200.

18. Shieh DH, Chiang LC, Lee CH, Yang YH, Shieh TY.

Effects of arecoline, safrole, and nicotine on collagen phagocytosis by human buccal mucosal fibroblasts as a possible mechanism for oral submucous fibrosis in Taiwan.

Journal of oral pathology & medicine. 2004;33(9):581-7.

19. Khan S, Chatra L, Prashanth SK, Veena KM, Rao PK.

Pathogenesis of oral submucous fibrosis.

Journal of cancer research and therapeutics. 2012 Apr 1;8(2):199.

20. Rajendran R.

Oral submucous fibrosis.

Journal of Oral and Maxillofacial Pathology. 2003;7(1):1.

21. Haider SM, Merchant AT, Fikree FF, Rahbar MH.

Clinical and functional staging of OSF.

British Journal of Oral and Maxillofacial Surgery 2000;38:12-5.

22. **Wang YY, Tail YH, Wang WC, Chen CY, Kao YH, Chen YK, Chen CH.**

    Malignant transformation in 5071 southern Taiwanese patients with potentially malignant oral mucosal disorders.

    BioMed Central oral health. 2014;14(1):99.

23. **Aggarwal A, Shetti A, Keluskar V, Bagewadi A.**

    Estimation of serum beta carotene levels in patients with oral submucous fibrosis in India.

    Journal of oral science. 2011;53(4):427-31.

24. **Angadi PV, Krishnapillai R.**

    Evaluation of PTEN immunoexpression in oral submucous fibrosis: role in pathogenesis and malignant transformation.

    Head and neck pathology. 2012;6(3):314-21.

25. **Yang JS, Chen MK, Yang SF, Chang YC, Su SC, Chiou HL, Chien MH, Lin CW.**

    Increased expression of carbonic anhydrase IX in oral submucous fibrosis and oral squamous cell carcinoma.

    Clinical Chemistry and Laboratory Medicine 2014;52(9):1367-77.

26. **Moutasim KA, Jenei V, Sapienza K, Marsh D, Weinreb PH, Violette SM, Lewis MP, Marshall JF, Fortune F, Tilakaratne WM, Hart IR.**

Betel-derived alkaloid up-regulates keratinocyte alphavbeta6 integrin expression and promotes oral submucous fibrosis.

The Journal of pathology. 2011;223(3):366-77.

27. **Ranganathan K, Kavitha R, Sawant SS, Vaidya MM.**

Cytokeratin expression in oral submucous fibrosis–an immunohistochemical study.

Journal of oral pathology & medicine. 2006;35(1):25-32.

28. **Nanda KD, Ranganathan K, Devi U, Joshua E.**

Increased expression of CK8 and CK18 in leukoplakia, oral submucous fibrosis, and oral squamous cell carcinoma: An immunohistochemistry study.

Oral surgery, Oral medicine, Oral pathology and Oral radiology. 2012;113(2):245-53.

29. **Zhou S, Li L, Jian X, Ou X, Jiang H, Yao Z, Xu C, Peng J.**

The phosphorylation of survivin Thr34 by p34cdc2 in carcinogenesis of oral submucous fibrosis.

Oncology reports. 2008;20(5):1085-91.

30. **Zhou S, Qu X, Yu Z, Zhong L, Ruan M, Ma C, Wang M, Zhang C, Jian X.**

Survivin as a potential early marker in the carcinogenesis of oral submucous fibrosis.

Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2010;109(4):575-81.

31. Kamat SS, Kumar GS, Koshy AV.

Immunohistochemical analysis of syndecan-1 in leukoplakia and oral submucous fibrosis.

Dental research journal. 2013;10(3):321.

32. Yadav A, Desai RS, Bhuta BA, Singh JS, Mehta R, Nehete AP.

Altered Immunohistochemical Expression of Mast Cell Tryptase and Chymase in the Pathogenesis of Oral Submucous Fibrosis and Malignant Transformation of the Overlying Epithelium.

PLoS ONE 9(5): e98719. doi:10.1371.

33. Ranganathan K, Kavitha R.

Proliferation and apoptosis markers in oral submucous fibrosis.

Journal of Oral and Maxillofacial Pathology 2011;15(2):148.

34. Humayun S, Prasad VR.

Expression of p53 protein and ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study.

National Journal of Maxillofacial Surgery. 2011;2(1):38.

35. Varun BR, Ranganathan K, Rao UK, Joshua E.

Immunohistochemical detection of p53 and p63 in oral squamous cell carcinoma, oral leukoplakia, and oral submucous fibrosis.

Journal of Investigative and Clinical Dentistry. 2014;5(3):214-9.

36. Tsai CH, Lee SS, Chang YC.

Hypoxic regulation of plasminogen activator inhibitor-1 expression in human buccal mucosa fibroblasts stimulated with arecoline.

Journal of Oral Pathology & Medicine 2014.

37. Anura A, Conjeti S, Das RK, Pal M, Bag S, Paul RR, Ray AK, Chatterjee J.

Computer-aided molecular pathology interpretation in exploring prospective markers for oral sub-mucous fibrosis progression.

Head & neck.2014. doi: 10.1002

38. Gurudath S, Ganapathy KS, Pai A, Ballal S, Asha ML.

Estimation of superoxide dismutase and glutathione peroxidase in oral submucous fibrosis, oral leukoplakia and oral cancer-a comparative study.

Asian Pacific Journal of Cancer Prevention. 2012;13(9):4409-12.

39. Pandiar D, Shameena PM.

Immunohistochemical expression of CD34 and basic fibroblast growth factor (bFGF) in oral submucous fibrosis.

Journal of Oral and Maxillofacial Pathology. 2014;18(2):155.

40. Löfstedt T, Fredlund E, Holmquist-Mengelbier L, Pietras A, Ovenberger M, Poellinger L, Påhlman S.

Hypoxia inducible factor-2α in cancer.

Cell Cycle. 2007 Apr 15;6(8):919-26.

Cancer research. 2002;62(5):1326-9.

41. **Keith B, Johnson RS, Simon MC.**

HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression.

Nature Reviews Cancer. 2012;12(1):9-22.

42. **Bertout JA, Majmundar AJ, Gordan JD, Lam JC, Ditsworth D, Keith B, Brown EJ, Nathanson KL, Simon MC.**

HIF2α inhibition promotes p53 pathway activity, tumor cell death, and radiation responses.

Proceedings of the National Academy of Sciences. 2009;106(34):14391-6.

43. **Imamura T, Kikuchi H, Herraiz MT, Park DY, Mizukami Y, Mino-Kenduson M, Lynch MP, Rueda BR, Benita Y, Xavier RJ, Chung DC.**

HIF-1α and HIF-2α have divergent roles in colon cancer.

International Journal of Cancer. 2009;124(4):763-71.

44. **Leek RD, Talks KL, Pezzella F, Turley H, Campo L, Brown NS, Bicknell R, Taylor M, Gatter KC, Harris AL.**

Relation of hypoxia-inducible factor-2α (HIF-2α) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in human breast cancer.

Cancer Research 2002; 62: 1326–1329.

45. Påhlman S, Lund LR, Jögi A.

Differential HIF-1α and HIF-2α Expression in Mammary Epithelial Cells during Fat Pad Invasion, Lactation, and Involution

PLoS ONE 10(5): e0125771

46. Bangoura G, Yang LY, Huang GW, Wang W.

Expression of HIF-2alpha/EPAS1 in hepatocellular carcinoma.

World Journal of Gastroenterology 2004;10(4):525-30.

47. Aruna DS, Prasad KV, Shavi GR, Ariga J, Rajesh G, Krishna M.

Retrospective study on risk habits among oral cancer patients in Karnataka Cancer Therapy and Research Institute, Hubli, India.

Asian Pacific Journal of Cancer Prevention 2011; 12(6): 1561-6.

48. Tilakaratne WM, Nissanka-Jayasuriya EH.

Value of HIF-1α as an independent prognostic indicator in oral squamous cell carcinoma.

Expert Review of Molecular Diagnostics 2011; 11(2): 145-147.

49. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, Harris AL

Hypoxia-inducible factors HIF-1α and HIF-2α in head and neck cancer relationship to tumor biology and treatment outcome in surgically resected patients.

Cancer Research. 2002; 62(9): 2493-7.

50. Chaturvedi P, Vaishampayan SS, Nair S, Nair D, Agarwal JP, Kane SV, Pawar P, Datta S.

Oral squamous cell carcinoma arising in background of oral submucous fibrosis: a clinicopathologically distinct disease.

Head & Neck. 2013;35(10):1404-9.

51. Balaji P, Govindraju P, Gupta A, Pawar Y, Gazge NM.

Oral Squamous Cell Carcinoma in Background of Oral Submucous Fibrosis: A Case Report.

International Journal of Scientific Study. 2015;1(12):40.

52. Massarelli E, Brown E, Tran NK, Liu DD, Izzo JG, Lee JJ, El-Naggar AK, Hong WK, Papadimitrakopoulou VA

Loss of E-cadherin and p27 expression is associated with head and neck squamous tumorigenesis

Cancer 2005; 103(5): 952-9.

53. Lee CH, Ko YC, Huang HL, Chao YY, Tsai CC, Shieh TY, Lin LM.

The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern Taiwan.

British Journal of Cancer. 2003; 88(3): 366-72.

54. Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, Lu YC, Li HF, Cheng AJ.

Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis.

Cancer Science. 2008; 99(8): 1507-14.

55. **Feller L, Lemmer J.**

Oral squamous cell carcinoma: Epidemiology, clinical presentation and treatment.

Journal of Cancer Therapy, 2012; 3: 263-268.

56. **Cruz I, Snijders PJ, Van Houten V, Vosjan M, Van der Waal I, Meijer CJ.**

Specific p53 immunostaining patterns are associated with smoking habits in patients with oral squamous cell carcinomas.

Journal of Clinical Pathology. 2002;55(11):834-40.

57. **Ho PS, Chen PL, Warnakulasuriya S, Shieh TY, Chen YK, Huang IY.**

Malignant transformation of oral potentially malignant disorders in males: a retrospective cohort study.

BioMedCentral cancer 2009;9(1):260.

58. **Fraisl P, Mazzone M, Schmidt T, Carmeliet P.**

Regulation of angiogenesis by oxygen and metabolism.

Developmental cell. 2009;16(2):167-79.

59. **Koukourakis MI, Bentzen SM, Giatromanolaki A, Wilson GD, Daley FM, Saunders MI, Dische S, Sivridis E, Harris AL.**
Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial.
Journal of Clinical Oncology. 2006;24(5):727-35.

60. **Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ.**
Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma.
Molecular and cellular biology. 2005;25(13):5675-86.

Annexures

ANNEXURE- I

RAGAS DENTAL COLLEGE HOSPITAL
(JAYA GROUP)
(Department of Oral & Maxillofacial Pathology)
2/102, East Coast Road, Uthandi, Chennai - 600 119, INDIA.
Tele : (044) 24530002-6-Exten. 33 Principal (Dir) 24530001 Fax : (044) 24530009

29.12.2015

TO WHOMSOEVER IT MAY CONCERN

From

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled **'STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) -2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS'** submitted by **Dr. I. Joseph** has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 5th May 2014.

RAGAS DENTAL COLLEGE Chennai

Dr. S. Ramachandran, M.D.S.,
IRB, Secretary,
Head of the Institution
Ragas Dental College& Hospital
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

ANNEXURE II

DISSERTATION PROTOCOL

1. **Title**

   Study of Hypoxia Inducible Factor (HIF) - 2 alpha expression in the malignant transformation of Oral submucous fibrosis (OSMF).

2. **Name and designation of the principal investigator**

   Dr. Joseph. I

   I year postgraduate student

   Department of Oral and Maxillofacial Pathology

3. **Name of HOD and staff in charge**

   Dr. K. Ranganathan , MDS, MS (Ohio), PhD

   Dr. K. Umadevi, MDS

   Dr. Elizabeth Joshua, MDS

   Dr. T. Rooban, MDS

4. **Department where the project is to be carried out**

   Study will be conducted in Department of Oral & Maxillofacial Pathology,

   Ragas Dental College & Hospital

5. **Duration of the project**

   1 year

6. **Background**

   HIF – 2 alpha are over-expressed in primary and metastatic human cancers, and the level of expression is correlated with tumor

angiogenesis and patient mortality. Molecules upregulated by hypoxia (HIF alpha) play a role in progression of fibrosis in OSMF.To ascertain the role of HIF – 2 alphaasa surrogate biomarker in the malignant transformation of Oral Submucous Fibrosis, a potential malignant disorder.

7. Hypothesis (null)

There is no difference in HIF -2 alpha expression in Oral Submucous fibrosis when compared to normal mucosa.

Aim

To study whether HIF-2 alpha expression is upregulated in oral submucous fibrosis and oral squamous cell carcinoma.

Objectives

1. To study the expression of HIF -2 alpha by Immunohistochemistry in archival blocks of:
    a) OSMF
    b) OSCC with areca nut habit
    c) OSCC without areca nut habit
    d) Normal mucosa
2. To compare the expression of HIF -2 alpha in OSMF, OSCC and normal oral mucosa by Immunohistochemistry.

8. Materials and methods

Sample:

Group I : 11 samples of OSMF

Group II : 15 samples of OSCC with areca nut habit

Group III : 15 samples of OSCC without areca nut habit

Group IV : 10 samples of normal mucosa

Procedure:

Immunohistochemistry (IHC)

Equipments and chemical reagents needed:

1. Microtome
2. Autoclave
3. Hot air oven
4. Slide warmer
5. Coupling jars
6. Measuring jar
7. Weighing machine
8. APES coated slides
9. Slide carrier
10. Aluminium foil
11. Micro-pipettes
12. Toothed forceps
13. Electronic timer
14. Beakers

15. Rectangular steel tray with glass rods

16. Sterile gauze

17. Cover-slips

18. Light microscope

Reagents used:

1. 1NHCl

2. 1N NaOH

3. APES (3 amino propyl ethoxysilaneeee)

4. Acetone

5. Tris EDTA buffer

6. Tris Buffer Saline with Tween

7. 3% H2O2

8. Deionized distilled water

9. Hematoxylin

10. Absolute alcohol

11. Xylene

Antibodies used:

1. Primary antibody – Anti-HIF-2-alphamouse monoclonal antibody- Clone ep-190b, Abcam (Annexure III)

2. Secondary antibody – Poly Excel-HRP Micro polymer IHC detectionsystem- Vkan Life Care (Annexure IV)

Statistics to be used:

- Chi-square test.

- Data analysis to be done using SPSS (Statistical Package of Social Science) version 21.
- Kappa analysis.

Signature of principal investigator

Signature of Head of Department

Remarks of committee:

Permission granted **YES** **NO**

Modifications / condition

ANNEXURE - III

abcam®
discover more

Anti-HIF-2-alpha antibody [ep190b] (HRP) ab81805

1 References 2 Images

Overview

Product name	Anti-HIF-2-alpha antibody [ep190b] (HRP)
Description	Mouse monoclonal [ep190b] to HIF-2-alpha (HRP)
Conjugation	HRP
Specificity	This antibody is specific for HIF-2-alpha and does not crossreact with HIF1 alpha.
Tested applications	WB, IHC-P
Species reactivity	**Reacts with:** Mouse, Rat, Human
Immunogen	Synthetic peptide corresponding to Human HIF-2-alpha aa 535-631. Sequence: MDGEDFQLSPICPEERLLAENPQSTPQHCFSAMTNIFQPLAPVAPHSPFL LDKFQQQLESKKTEPEHRPMSSIFFDAGSKASLPPCCGQASTPLSSM Run BLAST with NCBI Run BLAST with ExPASy
Positive control	IHC-P: heart, cardiac myocytes. WB: hypoxic A549 human lysate.
General notes	Ability to use ab81805 in mouse is mixed with some positive and some negative results.

Properties

Concentration	0.800 mg/ml
Form	Liquid
Storage instructions	Shipped at 4°C. Store at +4°C.
Storage buffer	Preservative: None Constituents: PBS
Purity	Protein G purified
Purification notes	Purified from mouse ascites.
Clonality	Monoclonal
Clone number	ep190b
Isotype	IgG1

Applications

Our Abpromise guarantee covers the use of **ab81805** in the following tested applications.
The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

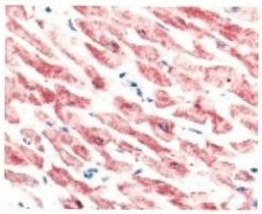
Application	Abreviews	Notes
WB		1/500. Detects a band of approximately 118 kDa (predicted molecular weight: 96 kDa).
IHC-P		1/150 - 1/300.

Target

Function	Transcription factor involved in the induction of oxygen regulated genes. Binds to core DNA sequence 5'-[AG]CGTG-3' within the hypoxia response element (HRE) of target gene promoters. Regulates the vascular

abcam®
discover more

Western blot - Anti-HIF-2-alpha [ep190b] antibody (HRP) (ab81805)



Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections) - Anti-HIF-2 -alpha [ep190b] antibody (HRP) (ab81805)

anti HIF2 alpha (unconjugated form of ab81805), at a 1/150 dilution, staining HIF2 alpha in paraffin embedded heart, cardiac myocytes by Immunohistochemistry.

ANNEXURE –IV

PathnSitu

Rev: A
Release Date: 03/13/2014
IVD

PolyExcel HRP/DAB Detection System
Universal kit for Mouse and Rabbit Primary Antibodies

Intended Use: For In Vitro diagnostic use

PolyExcel detection system is intended to use with primary antibodies raised against **mouse** and **rabbit** for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues or cell preparations.

Summary and Explanation: PathnSitu's highly sensitive and specific PolyExcel two step detection system is non-biotin, micro-polymer based detection system which significantly reduce or shows no back ground on tissues containing high levels of avidin, biotin ex: Kidney, Liver and lymphoid tissues. This system is based on an HRP labeled polymer, which is conjugated with secondary antibodies.

Principal of procedure: Incubating the specimen for 5–10 minutes with H_2O_2 quenches any endogenous peroxidase activity. The specimen is then incubated with respective diluted mouse or rabbit primary antibody, followed by incubation with the PolyExcel Target Binder for 10 minutes then followed by a PolyExcel HRP labeled polymer using recommended 10minutes incubation. Staining is completed by a 5–10 minute incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen which results in a brown-colored precipitate at the antigen site (DAB is a potential carcinogen; Please take appropriate precautions).

Kit Contents:

PathnSitu PolyExcel detection kit supplied as 3 pack sizes. Details below:

Description	Cat#/Pack Size	Kit Contents
PolyExcel HRP/DAB Detection System	PEH2-6ml	PolyExcel H_2O_2 PolyExcel Target Binder PolyExcel PolyHRP PolyExcel Stunn DAB Substrate Buffer PolyExcel Stunn DAB Substrate Chromogen
	PEH2-50ml	
	PEH2-100ml	

Materials required but not supplied:

1. Positive charged slides (PathnSitu Cat# PS011-72)
2. Control Tissues
3. Xylene
4. Isopropyl alcohol
5. DI Water
6. Hematoxylin

7. Cover glass
8. Mounting media
9. Antigen retrieval buffers (PathnSitu Cat# PS007, PS008, PS009)
10. Immuno wash Buffer (PathnSitu Cat# PS006)

Storage and Stability:
Store all reagents at 2°C to 8°C. Do not use after expiration date printed on vial. If reagents are stored under conditions other than those specified in the package insert, they must be verified by the user.

Protocol Recommendations:
Preparation of working solutions: DAB(DAB is a potential carcinogen; Please take appropriate precautions): In a1ml of Stunn DAB Buffer add 1drop of Stunn DAB chromogen. Mix well the preparation and store it in dark. This solution is stable for a week when stored at 2°-8°C. Always prepare fresh for clean and crisp results.

Deparaffinization:

1. Deparaffinize tissue sections in 3 changes of xylene.
2. Hydrate slides in a series of graded alcohols to water.

Pretreatment Solution/Protocol: Please refer to the respective primary antibody datasheet for recommended pretreatment solution and protocol.

Staining protocol* :(* Wash tissue sections with immuno wash buffer after every incubation).

3. **Peroxide Block:** Block for 5 minutes with PolyExcel H2O2.
4. **Primary Antibody:** Please refer to the respective primary antibody datasheet for incubation time and temperature.
5. **PolyExcel Target Binder:** Cover the tissue sections with PolyExcel Target Binder and Incubate for 10minutes at RT.
6. **PolyExcel PolyHRP:** Cover the tissue sections with PolyExcel PolyHRP and incubate for 10min at room temperature.
7. **PolyExcel StunnDAB**: Cover the tissue sections with StunnDAB working solution (please refer to preparation section on preparation of working solution) and incubate it for 5 min at room temperature.
8. **Hematoxylin:** Cover the tissue sections with Hematoxylin and incubate for appropriate time at room temperature.
9. Dehydrate slides through graded alcohols and Xylenes then cover slip with appropriate mounting medium.

Protocol Notes: The optimum antibody dilution and protocols for a specific application can vary due to many factors. These include, but are not limited to: fixation, incubation times, and tissue section thickness and detection kit used. The data sheet's recommendations and protocols are based on exclusive use of PathnSitu products. Due to the superior sensitivity of these unique reagents, the recommended incubation times and titers listed are not applicable to other detection systems, as results may vary. Ultimately, it is the responsibility of the investigator to determine optimal conditions.

Performance Characteristics: The protocols for a specific application can vary. These include, but are not limited to: fixation, heat-retrieval method, incubation times, and tissue section thickness and detection kit used. Due to the superior sensitivity of PathnSitu reagents, the recommended incubation times and titers listed are not applicable to other detection systems, as results may vary. The data sheet recommendations and protocols are based on exclusive use of PathnSitu products. Ultimately, it is the responsibility of the investigator to determine optimal conditions. These products are tools that can be used for interpretation of morphological findings in conjunction with other diagnostic tests and pertinent clinical data by a qualified pathologist.

Quality Control: PathnSitu follows and recommends to refer CLSI Quality Standards for Design and Implementation of Immunohistochemistry Assays; Approved Guideline-Second edition (I/LA28-A2). CLSI

US Office: 538, Selby Lane, Livermore, CA- 94551 USA, Ph: +1 925-218-6939
Corporate Office:CDC Towers, 3rd Floor, B-Block, Plot 10/8, Nacharam IDA, Road #5, Nacharam, Hyderabad-76, India.
Phone: 040-27015544/33,Fax:040-2701 5544 ,
E-Mail:customerservice@pathnsitu.com**Web:** www.pathnsitu.com

ANNEXURE V

DEPARTMENT DECLARATION FORM

The study title **"STUDY OF HYPOXIA INDUCIBLE FACTOR (HIF) – 2 ALPHA EXPRESSION IN THE MALIGNANT TRANSFORMATION OF ORAL SUBMUCOUS FIBROSIS"** has been done under the guidance of the staffs of the Department of Oral Pathology and Microbiology during my post-graduation during 2013- 2016. The same has been submitted as a part of the syllabus MDS degree programme in Oral Pathology and Microbiology of the Tamil Nadu Dr. M.G.R. Medical University. I shall publish in full or part of this work in any media only with the prior written approval of the head of the department.

Joseph. I
Post-graduation 2013-2016
Department of Oral and Maxillofacial Pathology

ANNEXURE VI

DECLARATION OF PLAGIARISM CHECK

FROM

Joseph. I
III-Postgraduate student
Department of Oral and Maxillofacial Pathology
Ragas dental college and hospital
Chennai.

TO

The Head of the Department
Department of Oral and Maxillofacial Pathology
Ragas dental college and hospital
Chennai.

SUB: Declaration of plagiarism check of my dissertation to be submitted to "The Tamil Nadu Dr. M.G.R Medical University" – April 2016

I hereby declare that I have checked my dissertation for plagiarism using "small SEO tools"- plagiarism checker software on 30.12.2015 date for this dissertation. The unique content was 75% and the plagiarism content was 25%. The plagiarism content corresponds to definitions and terminologies that have to be quoted.

Yours sincerely,

ANNEXURE VII

ABBREVIATIONS

1. OSCC	-	OralSquamous cell carcinoma
2. OSMF	-	Oral submucous fibrosis
3. HIF	-	Hypoxia-inducible factor
4. VEGF	-	Vascular endothelial growth factor
5. ARNT	-	aryl hydrocarbon receptor nuclear translocator
6. HRE	-	Hypoxia response elements
7. H & E	-	Hematoxylin and eosin
8. IHC	-	Immunohistochemistry
9. RNA	-	Ribonucleic acid
10. LM	-	Light microscope
11. APES	-	Amino propyl triethoxysilane
12. EDTA	-	Ethylene Diamine Tetra Acetate
13. HRP	-	Horse radish peroxide
14. DAB	-	Diaminobenzidine
15. DPX	-	Dibutyl phthalate in Xylene
16. MLI	-	Mean Labelling Index
17. OSF	-	Oral submucous fibrosis
18. HLA	-	Human Leucocyte Antigen
19. MMP	-	Matrix metalloproteinase
20. TIMP	-	Tissue inhibitors of matrix metalloproteinase

21. IL	-	Interleukin
22. TNF	-	Tumor necrosis factor
23. TGF	-	Transforming growth factor
24. ECM	-	Extra- cellular matrix
25. PCP	-	Procollagen C-proteinase
26. BMP	-	Bone morphogenetic protein
27. PNP	-	Procollagen N-proteinase
28. LOX	-	Lysyl oxidase
29. LTQ	-	Lysine tyrosylquinone
30. PAI	-	Plasminogen activator inhibitor
31. Plg	-	Plasminogen
32. tPA	-	Tissue plasminogen activator
33. Ig	-	Immunoglobulin
34. CD	-	Cluster of differentiation
35. NK	-	Natural killer
36. ROS	-	Reactive oxygen species
37. LPO	-	Lipid peroxidation
38. PTEN	-	Phosphatase and tensin homologue
39. PI3K	-	Phosphotidyl inositol-3-kinase
40. BCL	-	B Cell lymphoma
41. FAK	-	Focal Adhesion Kinase
42. ERK	-	Extracellularsignal-regulated kinases
43. MAPK	-	Mitogen-activatedprotein kinases

44. CAIX(CA9)	-	Carbonic anhydrase IX
45. TERT	-	Telomerase Reverse Transcriptase
46. CK	-	Cytokeratin
47. PanCK	-	Pancytokeratin
48. HMWCK	-	High molecular weight cytokeratin
49. MC	-	Mast Cell
50. MIB1	-	Mindbomb E3 ubiquitin protein ligase 1
51. BAX	-	BCL2-associated X protein
52. VEGFR	-	Vascular endothelial growth factor receptor
53. E-SOD	-	Erythrocytesuperoxide dismutase
54. GPx	-	Glutathione peroxidase
55. FGF	-	fibroblast growth factor
56. EGFR	-	epidermal growth factor receptor
57. PDGF	-	platelet-derived growth factor
58. bFGF	-	basic fibroblast growth factor
59. EPAS-1	-	endothelial PAS domain protein 1
60. mAB	-	monoclonal antibody
61. KRAS	-	Kirsten rat sarcoma viral oncogene homolog
62. pVHL	-	von Hippel–Lindau protein
63. ccRCC	-	clear cell renal cell carcinoma
64. TAM	-	tumor-associated macrophages
65. MNT	-	MAX network transcriptional repressor
66. JNK	-	c-Jun N-terminal kinase

67. BNIP3	-	BCL2/adenovirus E1B 19kDa interactingprotein 3
68. SOD1	-	superoxide dismutase 1
69. DLL4	-	delta-like ligand 4