"EXPRESSION OF FIBROBLAST ACTIVATION PROTEIN-ALPHA IN ORAL SQUAMOUS CELL CARCINOMA & ITS ASSOCIATION WITH LYMPH NODE METASTASIS- AN IMMUNO-HISTOCHEMICAL STUDY"

By

REG NO. IH0220003

Dissertation

Submitted to KLE Academy of Higher Education and Research

(KAHER), Belagavi

In Partial Fulfillment of the Requirements for the Degree Of

MASTER OF DENTAL SURGERY

In

ORAL & MAXILLOFACIAL PATHOLOGY & ORAL MICROBIOLOGY

(BRANCH - IV)

DEPARTMENT OF ORAL & MAXILLOFACIAL PATHOLOGY & ORAL MICROBIOLOGY

KAHER'S KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES, KAHER, NEHRU NAGAR, BELAGAVI -10, KARNATAKA.

2020 -2023

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA

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ABBREVATIONS

ABBREVIATIONS	FULL FORM
OSCC	Oral squamous cell Carcinoma
AJCC	American Joint Committee on Cancer
WDSCC	Well differentiated squamous cell carcinoma
MDSCC	Moderately differentiated squamous cell carcinoma
PDSCC	Poorly differentiated squamous cell carcinoma
TME	Tumour micro environment
CAF	Cancer associated fibroblast
EMT	Epithelial to mesenchymal transition
End-MT	Endothelial mesenchymal transition
CAF-S1	Cancer associated fibroblast - subset 1
DPP4	Dipeptidyl Peptidase 4
FAP	Fibroblast Activation Protein-α
MCP	Monocyte chemoattractant protein
PDAC	Pancreatic ductal adenocarcinoma
LNM	Lymph node metastasis
BM	Basement Membrane
LOX	Lysl Oxidase
SRC	Proto-oncogene tyrosine protein kinase
RAS-ERK	Rat Sarcoma-Extracellular signal regulated Kinase

GDF15-Akt pathway	Growth Differentiation Factor-15-AK Strain transforming
TNF-a	Tumor Necrosis Factor-α
(STAT3)	signal transducer and activator of transcription
CXCL12	C-X-C motif chemokine ligand 12
Wnt	Wingless related integration site
OS	Overall Survival
HNSCC	Head & Neck Squamous Cell Carcinoma
CAR T cells	Chimeric Antigen Receptor T cells

ABSTRACT

Introduction: Oral carcinomas arises as a result of multiple molecular events. This can be either due to exposure to carcinogens present in the environment or genetic predisposition. Once the malignancy is set, histologically several structural alterations take place, both at cellular and tissue level. The tumor microenvironment plays a major role in survival and promotion of cancer cells. Interactions amongst these cellular and structural constituents of the microenvironment confers invasive capabilities to cancer cells and mediate their dissemination to far off locations via complex signalling cascades. Cancer associated fibroblasts/CAF constitutes the most common connective tissue cell in the given TME. It mediates promotion to growth and metastasis of tumor cells by secreting cytokines, growth factors, chemokines, mediating ECM remodelling along-with disruption of inflammatory reactions and immune surveillance pathways. Till date there are very few studies in the literature regarding FAP-a expression in OSCC and its association to histological parameters. Hence, our study aimed at evaluating an association between Expression of Fibroblast Activation Protein-Alpha in OSCC & Lymph Node Metastasis (LNM) and also other prognostic histological parameters.

Method: 90 Formalin Fixed Paraffin Embedded tissue blocks (FFPE) of histologically diagnosed radical neck dissection cases, of Oral cancer were included in our study. All lesion proper slides of each case were thoroughly scanned and assessed to identify FAP α expression in OSCC cells and CAF in connective tissue stroma under microscope at x40 magnification. Immunohistochemical expression of FAP- α marker was analysed and percentage of positivity, staining intensity and location of FAP α expression in OSCC tissues was identified.

Results: Our results revealed a statistically significant association of increase in FAP-α +ve CAFs and staining intensity of FAP-α in CAF with LNM (p=0.0300 & p=0.0180) in OSCC. We also noticed statistically significant association of increase in frequency of FAP- α positive CAFs with prognostic histopathological parameters such as Tumor budding activity (p=0.0230), Lympho-vascular invasion(p=0.0120) through Chi-square test.

Conclusion: This study is first of its kind to show significant association of Lymph Node metastasis with expression of FAP α in OSCC. Though there are several mechanisms of tumor progression, we would like to add that when FAP α +ve CAF are present in connective tissue stroma the tumor cells show more invasive capability with increase in Tumor budding activity and LVI which might play a role in LNM.

Key words: Oral Carcinoma, Fibroblast Activation Protein-Alpha Marker, Tumor microenvironment, Immunohistochemistry.

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INTRODUCTION

Cancer has become the most life-threatening disease worldwide. Oral cancer is ranked 6th amongst all types of cancer globally, with our country representing the largest number of oral cancer cases and a 3rd of the total burden of OSCC cases, present worldwide. Oral cavity cancer poses an alarming health concern for the regions exhibiting economic development.[1] India, yearly reports around 77,000 new cases and 52,000 deaths, which sums up to approximately a 4th of total global incidences.[2]

Increase in cases of oral cancer ponders an important concern in regard to community health.[3] In comparison to western countries, challenges faced for OSCC is significantly high in a country like India with about 70% of the cases being reported in their advanced clinical stages i.e., Stage 3-4 by AJCC. Because of late detection, the rate of prognosis is very poor, with 5-year survival rates mounting to around 20% only.[4] OSCC contributes remarkably to the spectrum of various cancers.

Oral carcinoma arises due to multiple molecular events. This can be either due to exposure to carcinogens present in the environment or genetic predisposition. Once the malignancy is set, histologically several structural alterations take place, both at cellular and tissue level. To assess these alterations researchers have proposed many grading systems and also many histological parameters which can have an impact in predicting the aggressive nature of the tumor and thereby prognostic outcome. The most commonly followed grading system is the Broder's Grading system where OSCC is graded as WDSCC, MDSCC, PDSCC and anaplastic tumors based on differentiation and maturation of keratin produced by the tumor cells.[4] Another

grading system according to Bryne's is by categorising morphological features such as degree of keratinisation of tumor cells, nuclear pleomorphism, number of mitoses in high power field, mode of invasion and inflammatory infiltration. Each of these criteria are scored according to definition by Annoreth et al.[5] ranging from values starting from 1 to 4 Thus, the above-mentioned grading systems are popular and followed worldwide, but was found to be not sufficient in assessing the aggressive nature of OSCC. Hence, researchers started exploring other histopathological parameters that can be assessed routinely during histopathological reporting of OSCC.

The TME also mediates essential tumor survival and promotion functions by conferring invasive capabilities to cancer cells and mediate dissemination from its original site to distant locations via complex metastatic signalling. Tumor cells can get dislodged throughout the body via blood, lymphatics but only in the of a congenial TME metastases can occur. CAF are most common stromal cells in the TME and re-organizes extracellular matrix by forming tumor islands, and facilitates metastasis of tumor cells by secreting various cytokines and influencing signalling pathways. Identifying these proteases and the way they modify their substrates is crucial in co-relating cellular biology, physiology and pathogenesis and in determining the potential of targeting these proteases as therapeutic drug targets in Cancer Associated Fibroblasts.

In tumors the most abundant connective tissue component, CAF are distinguished from different cell subtypes based on the presence of several stromal markers, including integrin β1, fibroblast activation protein, and αlpha smooth muscle actin.[7,8]

CAFs can have varied origin from different cell types present in the microenvironment.

Local fibroblasts which can undergo mesenchymal–mesenchymal transition, epithelial cells via EMT, endothelial cells undergoing end-MT and also from marrow of bone by hematopoietic stem cells.[9]

Cancer cells transform fibroblasts by the following 3 steps i) recruiting, ii) transforming to CAF and iii) maintenance following which, these activated fibroblasts in the form of Cancer associated fibroblasts release various signalling molecules which in turn favour the viability of malignant cells within the microenvironment and promote transformation in cell types of differing origin.[10]

Cancer associated fibroblasts facilitate re-modelling of the matrix by releasing fibronectin and collagen, producing metalloproteinases, and stimulating increase in Vascular endothelial growth factor levels.

Additionally, CAF creates pathways that enable the migration and spread of tumour cells.[11-16] By promoting the viability, maturation, and activation of CD 4 positive, CD25 positive, and T-lymphocytes, a particular subset in TME called CAF-Subset has shown to slow down the immune system. Breast cancer cells are encouraged to spread to the bone by the osteoblast cadherin pathway if the initial tumour is less than 2 cm in size and contains CAF-S1 cells in the connective tissue.[17]

"Dipeptidyl peptidase protein family" comprises of 4 enzymatic members:

1) Dipeptidyl peptidase 4 2) Fibroblast activation protein 3) Dipeptidyl peptidase 8 and 4) Dipeptidyl peptidase 9, all possessing an ability to hydrolyse prolyl bonds.

Cyclic nature of their proline residues, confers a special cleaving ability which makes this group of proteases compelling in many aspects of cellular biology and as therapeutic targets in certain pathologies [18,19]

The Dipeptidyl Peptidase 4 (DPP-4) is the most completely investigated, characterised, and prototypical member of this family. It has universally expressed features and has an impact on a variety of physiological and pathological processes through its ability to cleave bioactive peptides.[17] After seeing its capacities to degrade and deactivate glucagon-like peptide-1 in in vivo conditions, it was also identified as a therapeutic target for type 2 diabetes.

FAP constitutes a type II membrane-bound glycoprotein, belonging to the group of serine proteases and expressed by CAF in pathological states.

FAP has demonstrated to possess tumorigenic promoting activity, and show high upregulation in a number of cancers, but is absent in normal adult tissues. FAPα can cleave native ECM proteins, including collagen I & IV, fibronectin, laminin and gelatin. FAPα promotes cancer-associated fibroblasts to secrete MCP-1, mediating macrophage chemoattraction to the tumor microenvironment.

The extracellular matrix's main component, collagen fibres, gives cells and tissues their structural support. Important biochemical and biomechanical processes are regulated and influenced by extracellular matrix proteins, which bind to and produce growth factors and bioactive peptides.[20]

Since collagen fibres contain a significant number of Glycine-Proline residues, FAP can digest them. Collagen type 1 fibres are unwound as a result of partial digestion, such as that caused by MMP 1, which makes it easier for FAP to cleave

while Collagen type 1 cannot be broken down in its natural state[21,22] In vitro,[21] FAP can also cleave denatured collagen type 3. Tumorigenesis requires the deposition and destruction of collagen fibres during matrix remodelling.

Several studies have associated Fibroblast activation protein in promoting angiogenesis in tumor microenvironment.[23,24,25] Demonstration by dual inhibition of Dipeptidyl peptidase 4 and Fibroblast activation protein led to reduces tumor vascularization, but such reduction was not observed when Dipeptidyl peptidase 4, was inhibited alone[23], thus implicating the possibility of enzymatic activity of Fibroblast activation protein in promoting angiogenesis.

Fibroblast activation protein has been proven to be able to predict survival rates, with increased Fibroblast activation protein expression serving as a signal for poor survival in PDAC and oral, gastric cancer.

Fibroblast activation proteinα role in proliferation and migration of tumor cells is still contested. There are 2 main hypothetical views supporting their mechanism of action.

In-direct hypothesis (1^{st}): Fibroblast activation protein mediates ECM remodelling, and these induced modifications to the matrix are therefore accountable for the improved capacity for cell proliferation, according to the first indirect hypothesis. It is debatable whether Fibroblast Activation Protein controls ECM remodelling through enzymatic activity or non-enzymatic activity in relation to this concept.

Direct hypothesis (2^{nd}): The fibroblast activation protein is expressed, which modifies intracellular signalling networks and, in turn, affects cell cycle proliferation networks, increasing cell growth.

Immuno-expression of Fibroblast activation proteinα has been studied in ESCC and its relation with Lymph Node Metastasis was also evaluated, but has not been evaluated in OSCC.[26]

- Selection of surgical methods for treating oral cancer depends on whether the tumor is associated with Lymph Node Metastasis or not.
- To determine treatment plans and the patient's prognosis by accurately identifying the patient's lymph node metastatic status.
- A compelling need to identify pathological markers which can help selecting patients with a high risk of LNM.
- Such pathological markers could provide accurate clinical judgements which can be used for catering individual treatment strategies.

Hence with the above-mentioned concepts in regard to the action of Fibroblast activation protein alpha in regard to tumor growth and promotion, the aim of our study was to find an association between expression of FAP-α in Oral squamous cell carcinoma and Lymph node metastasis.

AIM AND OBJECTIVES

AIM:

Evaluation of immunohistochemical expression of Fibroblast Activation Protein Alpha in Oral Squamous Cell Carcinoma and its association with Lymph Node Metastasis

OBJECTIVES:

1. To assess the immunohistochemical expression of Fibroblast Activation Protein Alpha in Oral Squamous Cell Carcinoma.
2. To associate the immunohistochemical expression of Fibroblast Activation Protein Alpha with presence of Lymph Node Metastasis.
3. To associate immunohistochemical expression of Fibroblast Activation Protein Alpha with Clinicopathological Parameters.

REVIEW OF LITERATURE

I. Background of Oral Cancer:

The incidence of Head and Neck Squamous cell carcinoma cases are increasing year by year with an average of 6,00,000 reported cases annually worldwide.[27]Despite the development of advanced diagnostic and molecular techniques in cancer research, the prognosis of HNSCC still remains compromised. This can be attributed to the heterogeneity in source of origin of HNSCC. As these can arise from the mucosal lining of various anatomical sites in head and neck region mainly larynx, oral cavity, hypopharynx, nasopharynx etc.[28]However the triggering factors in initiation of HNSCC are various mainly contributing to tobacco habit, betel quid, areca-nut, Human papilloma virus (HPV), environmental factors, genetic instability etc. Moreover, as hypothesized in previous literatures the incidence of HNSCC has surpassed the incidence of cervical cancer by 2020.[28,29]Recently, research field have also noticed a shift in the site predominance of HNSCC from laryngeo-pharyngeal complex to oropharynx.[30]The possible reason could be due to increase in use of tobacco habit either in the form of chewing or non-chewing form being the common predisposing factor in etiology of OSCC.

OSCC also has a multifactorial origin and etiology and persists as the most commonly occurring mouth neoplasm as compared to other oral malignancies.[31]The primary reason could be due to delay in seeking treatment and lack of proper follow-up. As the etiology being multifactorial, though the most common etiology being associated with tobacco habit but not necessarily always be habit associated the other causes can be either due to trauma or genetic mutation. Though the site of OSCC is

easily visualizable by the patients and accessible by the surgeons, prognosis still remains poor. This found to be challenging for onco-biologists, clinicians and researchers to identify the factors which aids in predicting the prognosis of oral cancer.

II. Histologically Prognostic Factors of OSCC:

The clinicians usually predict prognosis by evaluating the standard TNM staging and other modern diagnostic technologies but the main key indicators are predicted by pathologists. Pathologists have identified numerous histopathological features in OSCC tissues which gives a hint about the prognostic status of the patient correlating with the clinical staging of the tumor like Perineural invasion (PNI), Lympho-vascular Invasion (LVI), Tumor budding(TB), Worst pattern of Invasion(WPOI),Stromal Response etc. Furthermore researchers have not failed in updating the identification of newer prognosis related histopathological features. The updated 8th edition TNM classification by AJCC added two parameters such as Depth of Invasion (DOI) and Extranodal extension to correlate with T and N factors which was proposed in 2017, published and implemented in the year 2020.[32]

Currently, surgery, chemotherapy, targeted medication therapy, and radiotherapy are the major strategies of addressing oral cancer. Whereas the prognosis is improved by these treatments, different morbidity and fatality rates are seen in oral cancer patients due to late discovery and distant metastases. The ECM and basement membrane/BM, coupled with endothelial cells, adipose cells, tumor-infiltrating immune cells, cancer-associated fibroblasts/CAF, pericytes, and a number of signalling chemicals contribute to tumour progression, making up the tumour microenvironment.

In addition to MMP and other enzymes that degrade and alter the extracellular matrix and basement membranes around them, cancer cells can secrete growth factors and cytokines such as interleukin 6, 1, transforming growth factorβ-1, 2, Fibroblast growth factor-2, and platelet derived growth factor.

III. Extracellular Matrix & role of Normal Fibroblasts in maintaining ECM

The connective tissue (C.T.) stroma's most prevalent cell type is the fibroblast. These cells create and restructure the matrix by secreting a variety of proteins, which are crucial for the homeostasis and function of healthy tissues[33].

Additionally, fibroblasts influence the generation of inflammatory mediators, the recruitment of immune cells, and the immune system's response to bacterial lipopolysaccharides.[34] Fibroblasts can exhibit a variety of transcriptional programmes that are collectively controlled by epigenetic alterations and local stimulations, depending on the site of origin and the condition of the stromal tissue type.[35] The placement and specificity of the fibroblast tissue affect how the surrounding matrix is configured. The expression and activity of adhesion molecules and matrix remodelling enzymes are so diverse and heterogeneous that they serve as a base for tissue-specific fibroblast cells to negotiate and move through the surrounding stromal tissue.[36]

Fibrillar collagens are composed of matrix proteins made by fibroblasts, such as collagen types 1, 3, and 5, proteoglycans, fibronectin, and other glycoproteins and fibrillar components. These proteins work together to form a 3-D network, which results in scaffolds that are osmotically active in the interstitial spaces of tissues[33,37]. By generating and secreting laminins, various forms of collagen, and other basement

membrane-related proteins, these cells also aid in the creation of the sub-epithelial and endothelial basement membranes.[38]

Fibroblasts can mediate interactions with the matrix surrounding them depending on where they are located by expressing different membrane protein complexes, such as adhesion and signalling molecules. Thus, these cells engage responses to generate and/or destroy certain structures and chemicals present in the matrix[39] in response to various types of stimuli they receive.

Fibroblasts contain a number of surface adhesion receptors, including integrins, Syndecan, and cadherins, which serve as catalysts for interactions with the matrix as well as other cells. It has been discovered that the integrins that bind to collagen and fibronectin in specific are crucial for altering the surrounding matrix.[33] One of the additional mechanisms of ECM remodelling is the production of matrix degrading and enzymes that manifest crosslinking.[40]

Pro-inflammatory cytokines and growth factors, including platelet-derived growth factors, fibroblast growth factors, transforming growth factor family members, and interleukin-1 and the other IL family members, control the expression of extracellular matrix modulators.[40,41].

The families of these proteolytic enzymes include type 2 transmembrane serine proteases, matrix metalloproteinases, cathepsins, urokinase-plasminogen system proteins, and others. [10].

Systemic inhibitors such 2-macroglobulin, which constitutively regulate tissue maintenance and repair, as well as tissue inhibitors of metalloproteinases and plasminogen activator inhibitor, limit the protease activities of these enzymes[10].

Fibroblasts promote the integrity of tissues and organs and give them the right surroundings in which they can carry out conventional tissue functions[43]. There is proof that damaging these structures is a necessary stage for the genesis and development of malignant tumours.

While the normal stroma confers tumor-restraining functions to limit tumour initiation and progression, some cancer cells can endure the suppression and begin to reconstruct and modify the TME into one that confers cancer-supporting functions..

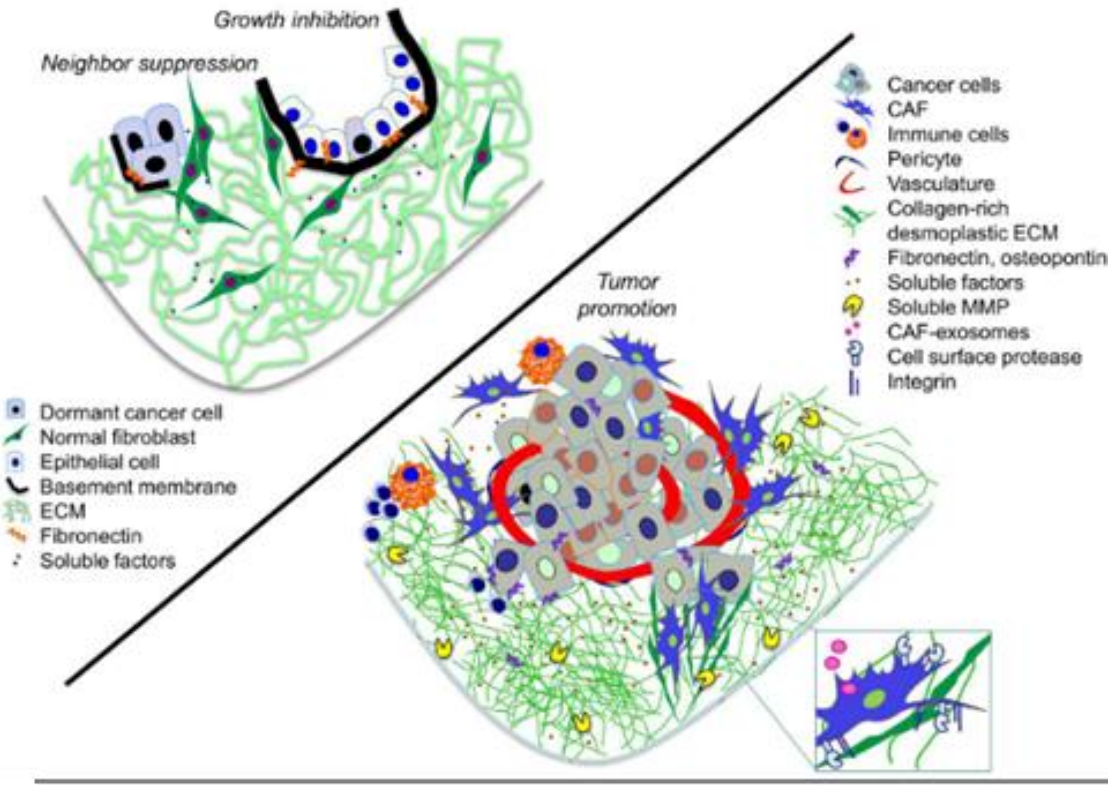


Figure 1: Photograph depicting the dual role of fibroblasts in Tumor Micro-environment Twana et al. Fibroblasts in the Tumor Microenvironment: Shield or Spear?

III.1. Extracellular matrix in Tumor Micro-environment (TME):

Similar to the role of stromal cells, detrimental roles are exerted by the non-cellular components of the Tumor Microenvironment which constitutes the Extracellular matrix, in favouring progression and metastasis of a tumor.

A network of extracellular proteins and other macromolecules known as the extracellular matrix contains several types of collagen, fibronectins, hyaluronan, elastins, integrins, and microfibrillar proteins which support the structure and biochemistry of the tissue. It is expressed in basement membranes and interstitial areas and governs cellular differentiation and proliferation while preserving tissue homeostasis.[44]

During commencement of tumorigenesis, it restrains malignant cells from proliferating and metastasizing by acting as a biological shield. However, during progression of a tumor, this extracellular matrix is re-modelled and transformed into a metastasis favouring microenvironment[45] usually referred to as Tumor Microenvironment. Such matrix rearrangements are accompanied by an overabundance of collagen fibre formation in the interstitial matrix, which then progresses to fibrosis, a symptom of different tumors.[46] This fibrotic reaction is primarily brought on by Cancer Associated Fibroblasts, which are the primary enzymes responsible for collagen synthesis.

In addition to collagen upregulation, these stimuli cause LOX to cross-link collagen fibres, which results in fibrosis and causes tumour stiffening14. Patients who appear with a higher connective tissue to fat ratio are also more likely to develop breast cancer[48,49].

Solid stress,[50] a term used to describe mechanical forces within the tumour that are mediating compression, has been calculated to reach a maximum of 142.4 mmHg, or 19.0 kPa, in humans. [51].

Malignant cells modify their cytoskeleton in response to mechanical compression, causing forces to be transferred to their surroundings. These matrix signals, which are transmitted amongst cells via mechano-sensors like cell adhesion matrix complexes and growth factors, might cause cancer cells to become overly sensitive.[50,52-58].

Solid stress has been observed to either initiate fibroblast activation or act directly on pancreatic cancer cells to promote their invasion [62,63]. Tumors with a dynamic stiffened matrix are often metastatic and correlate to poorer prognosis[47,53,59-61].

The primary treatment regimens for oral cancer consists of surgery, chemotherapy, radiotherapy & combined surgical and chemotherapy management. These treatments do improve the prognosis, but late discovery and distant metastases due to interaction of cancer cells with various components of the tumor micro-environment i.e., Cancer associated Fibroblasts contributes to differing morbidity and mortality rates in oral cancer patients. Cancer-associated fibroblasts being a major stromal component of the connective tissue stroma promote tumor proliferation and therapy resistance, and provide immune exemption to the tumor cells. There is increasing literature-based evidence that Cancer-associated fibroblasts play an important role in the growth and metastasis in many tumors of varying histology.

Therefore, understanding the interactions between Cancer-associated fibroblasts and tumor cells may contribute to the future development regarding cancer therapy. Therefore, it is vital to improve the accuracy of early diagnosis of oral cancer and to find potential factors that may serve as targets for improved surgical intervention & effective drug therapy.

Fibroblasts are frequently exposed to a variety of distinct stimuli in OSCC, which can encourage some distinctive traits in them.

As a result of the gene alterations required for the transformation from normal fibroblasts to Cancer associated fibroblasts which promote growth and invasion of tumours, the proliferative capability and autocrine signalling of cancer-associated fibroblasts may also increase.[180]

IV. Cancer associated fibroblasts (CAF):

Under physiologically normal circumstances, fibroblasts have a sedentary state.[177] The physiological condition of fibroblasts fluctuates in response to various stimuli, enabling them to become activated and exhibit infinite protein synthesis activity and contractile functions. Activated cells exhibit greater migratory capacity than normal fibroblasts and are more susceptible to genetic changes, allowing them to serve as progenitors for several cell types.[178,179]

CAF are considered as, all the fibroblasts found within and surrounding tumor tissues, which are activated from normal resident tissue fibroblasts or transdifferentiated from non-fibroblastic lineage cells due to the stimulation by TME[47,48].

Cancer-associated fibroblasts were thought to be tumor promoting agents by initiating building up and remodelling of ECM. Cancer-associated fibroblasts being a major stromal component of the C.T. stroma promotes tumor proliferation and therapy resistance, and provide immune exemption to the tumor cells.[70]

IV.1 Origin of CAF's:

Various theories have been put forward in regard to their origin

By upregulating Notch signalling, Strell, C. et al. shown in their work that interactions between cancer cells and fibroblasts increase the phenotype of cancer-associated fibroblasts in breast cancer.[71]

Procopio, M. G. et al however, Loss or downmodulating the Notch effector CSL, in dermal fibroblasts is sufficient for Cancer-associated fibroblasts activation and CSL functions as direct repressor of multiple senescence[72].

Numerous inflammatory modulators, such as interleukin-1 acting through nuclear factor kappa-B and interleukin-6 acting primarily on signal transducer and activator of transcription factors, can also encourage the activation of cancer-associated fibroblasts..[73,74]

In their cell culture study of fibroblasts exposed to the proinflammatory cytokine leukaemia inhibitory factor (LIF), Albrengues, J. et al. came to the conclusion that the exposure triggers an epigenetic switch that causes constitutive activation of the signal transducer and activator of transcription signalling, which results in fibroblast reprogramming into CAF, which encourages extracellular matrix remodelling and cancer cell invasion.

Crosstalk and positive feedback involving Janus kinase and signal transducer and activator of transcription signalling, can reprogram the cytoskeleton and result in histone acetylation hence promoting CAF activation.[75,76]

Physiological stress can be counted in as another contributing factor for stroma genesis. Heat shock factor 1 , which responds in part to protein misfolding, is also required for the generation of Cancer-associated fibroblasts.[77,78]

Amatangelo & Bassi, et al., and various other literature-based evidences have also confirmed the role of physical modifications in the matrix capable of initiating Cancer-associated fibroblast activation[79-86].

According to in vitro research, serum response factor-driven transcription as well as Yes associated protein 1 -TEAD-driven transcription can be activated by the induction of fibroblast stretching, which occurs from the hyperproliferation of transformed epithelial cells.[79-83]

A variety of genes encoding for connective tissue growth factor and cysteine-rich angiogenic inducer, as well as other genes linked with cancer-related fibroblast, are activated by cooperation between these transcription factors.[83].

Molecules, such as connective tissue growth factor and cysteine-rich angiogenic inducer 61, and the contractile cytoskeleton cooperate amongst themselves resulting in increasing stiffness of tissues, which further drives Serum response factor driven dependent and Yes associated protien1 dependent transcriptional programmes, locking Cancer-associated fibroblast into a self-sustaining positive-feedback loop.[83]

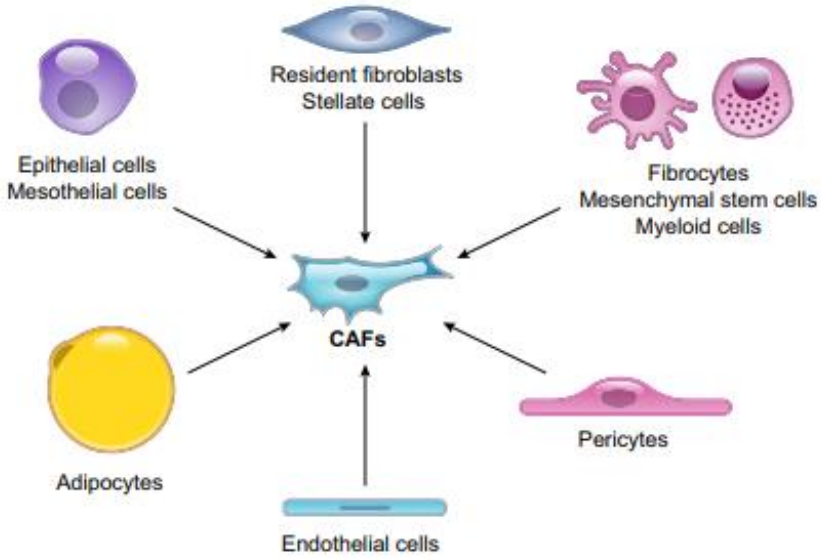


Figure 2: Various source of origin proposed in regard to formation of cancer-associated fibroblasts.

IV.1 ROLE OF CAF IN ECM:

CAFs also cater as a considerable source of GF, cytokines and secretory exosomes which promotes tumor growth and attune therapy responses[87,88-90].

Growth arrest-specific protein 6, fibroblast growth factor 5, growth differentiation factor 15, and hepatocyte growth factor, among others, are produced by cancer cells and encourage their proliferative and invasive behavior[91,92-95]. By offering a different BRAF-independent route for extracellular signal regulated kinase-Mitogen Activated Protein Kinase activation, hepatocyte growth factor has also been linked to the mediation of resistance to Proto-oncogene BRAF targeted therapy.[96]

Other elements of the microenvironment are influenced by the secretory components of cancer-associated fibroblast. Stromal cell production of vascular endothelial growth factor can promote angiogenesis[97,98].

Cytokines and chemokines which are released by Cancer-associated fibroblasts, act on a range of leukocytes, including CD8 positive T cells, regulatory T cells and macrophages, with both inducing immunosuppressive and immune-promoting consequences.[99]

Most people believe that IL-6, C-X-C chemokine ligand 9, and Transforming Growth Factor play established roles in suppressing T cell responses, which is one of the primary functions of cancer-associated fibroblasts.[100]

Cancer-associated fibroblasts have also been seen to cross-present antigens, which could activate CD4 positive T cells while suppressing CD8 positive T cells. Further supporting this inverse connection is clinical analysis.[101,102]

Interleukin-6 may also promote suppression of the immune system via its systemic effects on metabolism[104]. Along with targeting focal adhesion kinase in cancer cells, interference with C-X-C chemokine ligand 12 production by cancer-associated fibroblasts also encourages T cell-mediated tumour control[105,106,107] and lowers the activation rate of stromal fibroblasts, leading to the development of an immunosuppressive environment.[108].

Tumour necrosis factor produced by Cancer-associated fibroblasts has more meticulous effects on the immune response and with suppression of TNF signalling immunosuppressive activity of Fibroblast activation protein positive fibroblasts is highlighted[105,109,110]. Additionally, stromal fibroblasts interact with tumour cells via exchanging metabolites and amino acids with cancer cells and cancer-associated fibroblasts.[111-114]

Cancer-associated fibroblasts, demonstrating a matrix-producing and contractile phenotype or an immune modulating phenotype termed as ‘myo-CAFs’ and ‘i-CAFs’ , with the prefixes denoting their respective phenotypes have also been observed.

Pancreatic cancer cases have shown Cancer-associated fibroblasts most proximal to the cancer cells exhibits a myo-CAF phenotype, with increased Transforming Growth Factorβ driven αlpha smooth muscle actin expression and a contractile character[73,74,115].

I-CAF stands for distantly present cancer-associated fibroblasts that express greater quantities of interleukin-6. Transforming Growth Factor-mediated inhibition of the Interleukin 1 receptor, which triggers Nuclear Factor Kappa-B signalling and subsequent Interleukin 6 production, which also operates on Signal Transducer and activates transcription factors, explains the specificity between the two phenotypes.[73,74,115].

Breast cancer can also exhibit divergent Cancer-associated fibroblasts phenotypes, and the primary discriminating marker being Fibroblast activation proteinα. T In general agreement with tumour rejection seen after the eradication of fibroblast activation protein-expressing fibroblasts in experimental settings, fibroblast activation protein-expressing fibroblasts are associated with T-regulatory cell-mediated immune suppression and a poor clinical outcome[103,105].

IV.3. Role of Cancer-associated fibroblasts in progression of tumors:

The beginning of cancer invasion involves coordinated actions altered at the cancer cell surface by invasion promoting signals. This occurs through the epithelial and endothelial basement membranes.[117-119]

Willis, A.L & Sabeh F et al., concluded that tissue-invasive abilities can either proceed by protease-dependent or independent strategies whose selection is not only administered by the characteristics of the migrant cell population, but also by the structural properties of the intervening matrix.

By creating ECM tracks that allow for the collective invasion of cancer cells that have not fully undergone mesenchymal transition and subsequently adhere together by E-cadherin-mediated or possibly also other types of adhesion factors at the primary tumours, CAFs also serve as guides for stromal dissemination.[83,84]

The metastatic cascade is also influenced by extensive changes in the tumour microenvironment and matrix breakdown caused by MMPs released by cancer-associated fibroblasts. The production and activation of matrix metallo-proteinases 1, 2, 3, and 9 are examples of such tumor-inducing proteolytic processes by Cancer-associated fibroblasts which in turn mediates disruption in polarity and architecture of the tissues, as well as enhancing the ability of cancer cells to downregulate adhesion and navigate through the stromal containments.[123-128]

Matrix metallo-protienases inhibitor i.e., Tissue inhibitors of metalloproteinases 1 overexpression in Cancer-associated fibroblasts however has also been observed to support progression of prostate and colon cancer in in-vivo conditions[129]. However, exhausting all the members of the tissue inhibitors of

metalloproteinases family in fibroblasts enhances cell motility and cancer stem cell-like properties in breast cancer[129]. Such complete Tissue inhibitors of metalloproteinases 1 inactivation is sufficient for Cancer-associated fibroblast activation.

It has been discovered that secretory exosomes generated by cancer-associated fibroblasts facilitate and improve the ability of gastric cancer cells to invade by increasing the expression of matrix metallo-proteinases 2 in the same.[130] Breast cancer cells' growth and stem-like properties are fueled by the activation of Notch signalling and RhoA by exosomes carrying dis-integrin and metalloproteinase10. It has also been demonstrated that inactivating p53 and Notch effector stimulates the growth of cancer cells and fibroblasts linked with cancer.[131,132]

Additionally to, remodelling of extracellular matrix structures, Cancer-associated fibroblasts also secrete numerous proteins, serving as a link between the stromal matrix and cancer cells, with alteration of various cancer cell signalling cascades, hence aggravating invasion and metastasis[133]. One of those protein Fibroblast activation protein-α is overregulated in many tumor tissues, which includes breast[134-137], colorectal[138-140] pancreatic[141-144], lung[145-147], brain[148-150], intrahepatic bile duct[151], and ovarian[152-155]cancers.

- Fibroblast Activation Protein was examined by Wu et al. in their work, which also addressed the mechanism through which this protein encourages mesenchymal transition in OSCC. They looked at proteins that interact with fibroblast activation protein during the mesenchymal development of OSCC and discovered that this might be done by non-enzymatically downregulating dipeptidyl peptidase 9. Fibroblast Activation Protein, which has pro-

tumorigenic action, is openly expressed in fibroblasts linked to cancer. This protein has been found to interact intracellularly with dipeptidyl peptidase 9. In cancer tissue, DiPP9 protein and mRNA levels were downregulated. Low expression of dipeptidyl peptidase 9 was associated with poor survival rates for those with oral cancer. Downregulation of Dipeptidyl Peptidase 9 promotes cancer cell growth.

- It has been demonstrated that inhibiting fibroblast activation protein lowers cell proliferation and metastasis in oral cancer via the phosphatase and tensin homolog, phosphoinositide 3-kinase, and rat sarcoma viral oncogene homolog-mitogen-activated protein kinase pathways. Fibroblast Activation Protein suppression reduces the propensity for cell invasion and migration. Fibroblast Activation Protein expression is downregulated or momentarily silenced, which lowers cell proliferation in vitro and prevents the development of cancer xenografts in vivo. Fibroblast Activation Protein deficiency reduces cell migration and its capacity for invasion.

A number of intracellular and extracellular cell surface proteins have been used to isolate or identify Cancer-associated fibroblasts to understand their role in tumor biology. One amongst these proteins “Fibroblast activation protein” FAP-A has been studied in various carcinomas but has not been extensively studied in OSCC tissues.

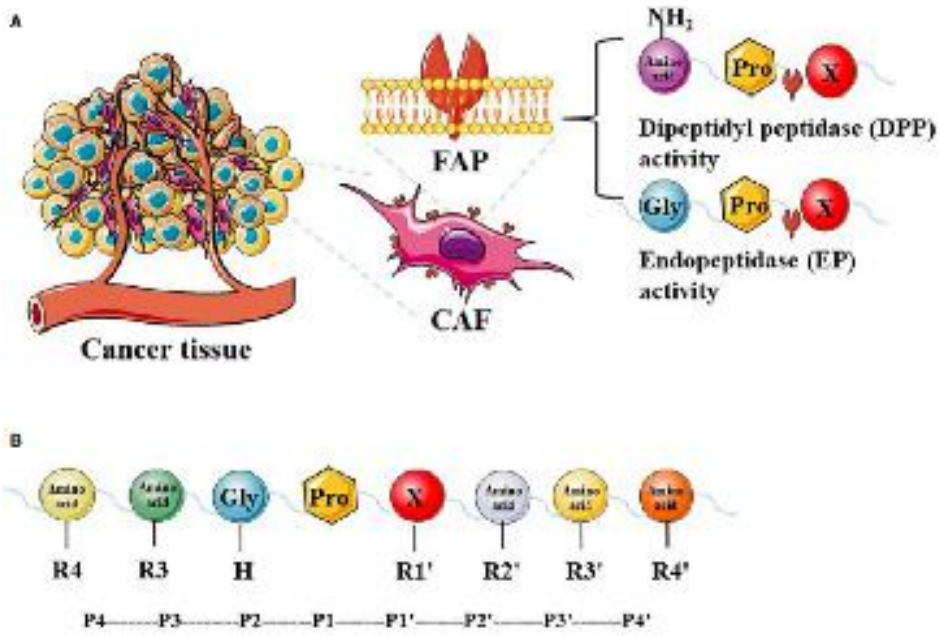


Figure 3: FAP expression on CAF

V. FAP-alpha

Rettig et al.[156] found out FAPα using a monoclonal antibody,F19 in the year of 1986, which showed reaction towards activated fibroblasts in in-vitro settings.

FAP protein was then eventually described by Rettig et al. as an antigen expressed on malignant epithelial cell surface, sarcomas, granulomas, and primitive mesenchymal fibroblasts. It was given the moniker "Fibroblast activation protein-" because it did not express itself on either benign tumour cells or normal fibroblasts.[156]

Although later sequence analyses of their protein structures showed that FAP and seprase are the same proteins [159,160], it is a membrane-bound gelatinase dimer that was also found at the invasive front of the human melanoma cell line LOX by Aoyama et al.[157] and named "seprase" by Monsky et al.[158] due to its surface expression character trait..

Fibroblast activation protein expression is not seen in systemically healthy adult tissues of mammals, according to a later study by Busek et al., [161] but some Fibroblast activation protein-positive cells were found in the placental tissue and uterine stroma, particularly during its proliferative phase[162], some foetal tissues[162], and multipotent stromal cells.[163].

(ii) STRUCTURE & FUNCTION of FAP α:

A 170-kda homo-dimer with "2 N-terminally glycosylated subunits" makes up the fibroblast activation protein. It belongs to the prolyl peptidase family, which also includes Dipeptidyl peptidase-4, Dipeptidyl peptidase-7, Dipeptidyl peptidase-8, Dipeptidyl peptidase-9, and Prolyl carboxypeptidase. It is a type II transmembrane serine protease.

The Fibroblast activation protein and Dipeptidyl peptidase proteins share 70% homology in their amino acid sequence[164] and also in their serine, aspartic acid, and histidine residues form a catalytic triad. [165].

The Fibroblast activation protein gene location has been tracked on to chromosome 2q23 and comprises of 26 exons with a combined size of 73 kb with some authors believing that FAP might have evolved from duplication of the Dipeptidyl peptidase-4 gene.

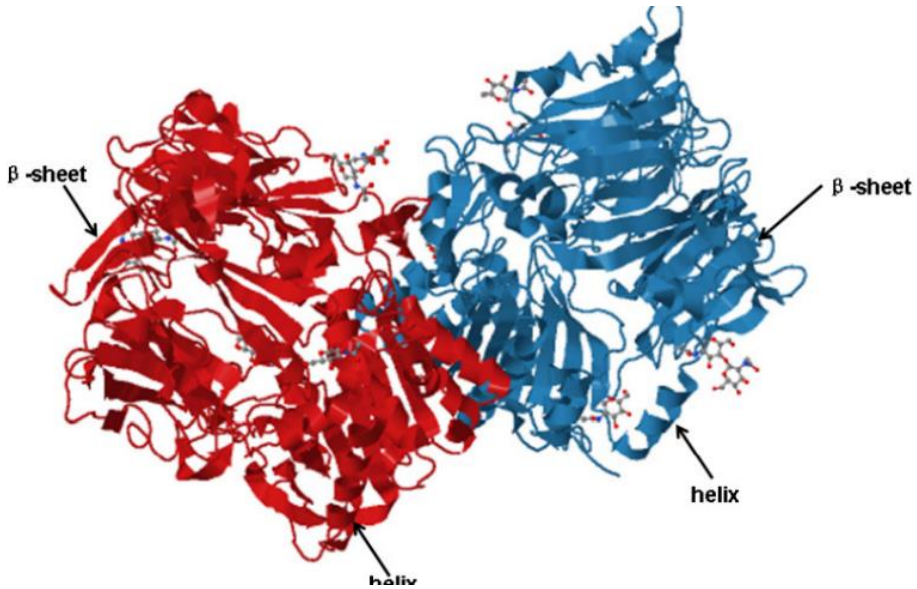


Figure 4: Cartoon representation of the Fibroblast activation protein -α homodimer structure comprised of Helix & B-sheets.

PHYSIOLOGIC PROPERTIES OF FIBROBLAST ACTIVATION PROTEIN

Enzymatic and Non enzymatic Activity and Substrates of Fibroblast activation protein

Fibroblast activation protein expression is seen in various animals, which include mice[166-168] and xenopus[168].

The mouse fibroblast activation protein gene, which is found on chromosome 2, has 26 exons and a total length of 60 kb, making it essentially identical to the human fibroblast activation protein gene. For preclinical research on therapies that aim to target Fibroblast activation protein, mouse models are so chosen.

Due to research that screened known Dipeptidyl peptidase IV substrates for cleavage by Fibroblast activation protein due to their somewhat comparable structural homology, some of the Fibroblast activation protein's known substrates have been discovered.

Studies have demonstrated that Fibroblast activation protein unique dipeptidyl peptidase activity allows it to cleave neuropeptide Y, peptide YY, substance P and brain natriuretic peptide 32[169].

Denatured collagen types 1 and 3 [170,171], α2 antiplasmin cleaving enzyme, and the recently identified fibroblast growth factor 21 are known substrates of FAP's endopeptidase activity.[172].

In the course of tissue repair, where a clot is formed by the deposition of fibrin, FAP's capacity to cleave 2 anti-plasmin has been extensively studied. Through a natural process known as fibrinolysis, the created clot is broken down by plasmin, causing the scar to disappear. Since anti-plasmin 2 inhibits plasmin, the rate at which the fibrin clot dissolves is decreased. When FAP cleaves 2-antiplasmin, it produces a more powerful inhibitor of plasmin.[173].

Non-Enzymatic activity of Fibroblast activation protein has been demonstrated with studies on mutated Fibroblast activation protein i.e., Fibroblast activation protein with defective enzymatic activity which has revealed that it may also have non-enzymatic functions.

Studies showing that nonenzymatically active Fibroblast activation protein reduced the tumorigenicity of mouse melanoma cell lines while normal enzymatically active Fibroblast activation protein increased tumorigenicity suggesting that lower Fibroblast activation protein enzymatic activity may exert beneficial biological effects.[174]

Similar research on breast cancer cell lines that had been transfected with a fibroblast activation protein variant with the least amount of enzymatic activity demonstrated rapid tumour growth in vivo and ECM distortion as compared to un-transfected cell lines[175]. Fibroblast activation protein can promote tumour growth and extracellular matrix breakdown irrespective of its high or low enzymatic activity.

Similar study on fibroblast cells transfected with enzymatically incompetent FAP displayed increase in growth and migration in cell lines of breast cancer, with Fibroblast activation protein activating PI3Ks pathway and signalling pathways for MMP 2 and 9[176].

Studies on oral cancer cells have also indicated that Fibroblast activation protein was overly expressed, and knockout of Fibroblast activation protein gene in cells inhibited tumor cell proliferation, migration by inhibiting the PTEN homolog, PI3KB and Ras-ERK signalling pathways[177].

Through interactions with Dipeptidyl peptidase IV, MMP 1 and 2, urokinase-type plasminogen activator, and other proteins, fibroblast activation protein may help promote inter-cell signal transduction pathways to facilitate tumour cell invasion.[178,179].

Fibroblast activation protein and integrin together decreased RhoA activity and increased the migration of mesenchymal stromal cells generated from bone marrow. [180].

Inflammatory cytokines like Interleukin-1b, Transforming Growth Factor-b, and Tumor Necrosis Factor-a can encourage the migration of bone marrow-derived mesenchymal stromal cells by overexpressing Fibroblast activation protein. In

fibroblasts, fibroblast activation protein favourably activates signal transducer and transcription activator.[181]

Signal transducer and activator of transcription factor and C-C Motif Chemokine Ligand 2 levels are positively linked with the expression of the fibroblast activation protein. Because the PI3K and Sonic Hedgehog pathways are activated in cells that overtly express Fibroblast Activation Protein, these cells have more capacity for proliferation and migration.[181]

Given the context, phosphoinositide 3-Kinase and sonic hedgehog pathway inhibitors may prevent Fibroblast activation protein over-expression and thereby inhibit tumour cell proliferation and migration. When Fibroblast Activation Protein forms a compound with the FAK protein, its phosphorylation is lowered, which may eventually encourage Fibroblast Activation Protein overexpression..[178].

FIBROBLAST ACTIVATION PROTEIN EXPRESSION IN DIFFERENT TUMOR TYPES

Normal tissues don't have Fibroblast activation protein expression.

However, FAP overexpression can be noticed in many tumor tissues, including breast[134-137], colorectal[138-140], pancreatic[141-144], lung[145-147], brain[148-150], intrahepatic bile duct[151], ovarian[152-155], gastric cancers.

Fibroblast activation protein expression has been found to be elevated in several tumours having non-epithelial origins, including melanoma[182,183] and myeloma [184], in addition to the wide spectrum of histological predominance. Because of the frequent interstitial overexpression of fibroblast activation protein in various malignancies, this protein is regarded as a universal marker for fibroblasts associated with cancer.

FIBROBLAST ACTIVATION PROTEIN'S ROLE IN TUMORS

The expression of Fibroblast activation protein in various tumors has initiated many studies to assess its role regarding the pro-tumor and anti-tumor effects of its expression.

The combined findings have shown that Fibroblast activation protein expression has significant implications on tumour growth by affecting invasion and proliferation of cells, mediating vasculogenesis and EMT, and supporting immunosuppression and treatment resistance.

Facilitating Tumor Cell Proliferation and Invasiveness:

Fibroblast Activation Protein, according to the findings of numerous research, mediates the proliferation, migration, and invasion of tumour cells, which eventually results in the development of tumours.

The two primary theories for the fundamental processes of FAP are[158,175-180,185,186]

1. The 1st hypothesis states that by an indirect mechanism Fibroblast activation protein regulates extracellular matrix remodeling leading to rise in tumor growth and invasion[158,175-177]. It still remains questionable whether Fibroblast activation protein regulates matrix remodeling via its enzymatic or non-enzymatic abilities.
2. The second idea suggests a direct mechanism by which the fibroblast activation protein's synthesis influences signalling pathways that control cell cycle and proliferation, ultimately boosting tumour growth..[178-180,185-187].

The 1st hypothesis has been supported by many studies[158,175,176].

In accordance with the second concept, cell cycle arrest is caused by transfecting cell lines with a short interfering RNA that targets the fibroblast activation protein in ovarian cancer associated fibroblasts.

Lung cancer cell lines have elevated levels of fibroblast activation protein, which controls cellular proliferation, tumour cell motility, and invasion. The protein kinase B and sonic hedgehog signalling pathways are also increased in conjunction with this.[187].

The relevance of fibroblast activation protein in upregulating the phosphatase and tensin homolog, protein kinase B, and Ras-ERK signalling pathways has been suggested by a small number of research on oral squamous cell carcinoma[156].

The results of co-culturing pancreatic ductal adenocarcinoma cell lines with fibroblast activation protein-positive fibroblasts were examined in this study by Kawase et al. in which they discovered that increased phosphorylation of tumour suppressor genes in cancer cells encourages cell cycle progression and proliferation.[186]

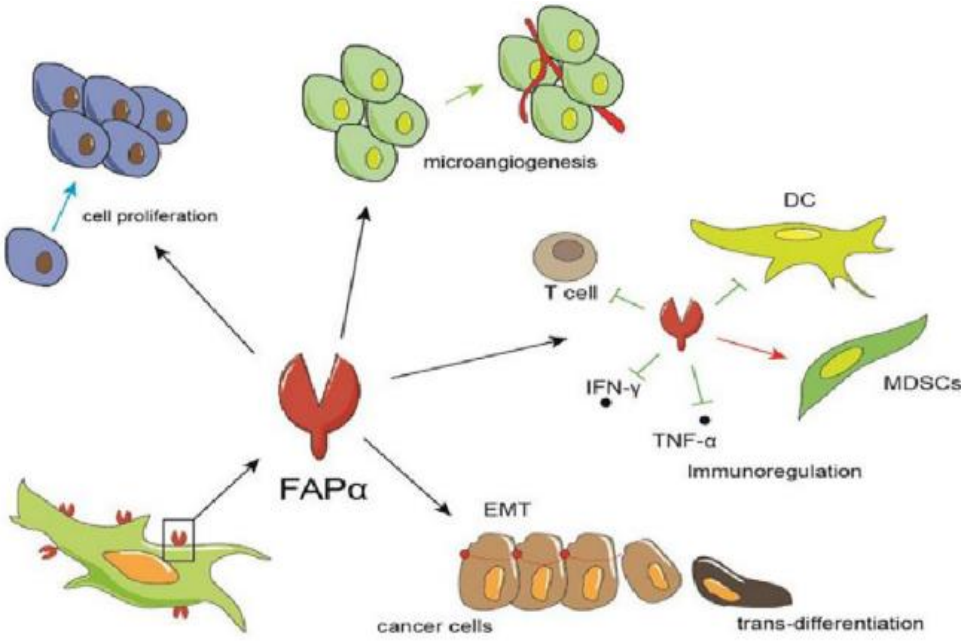


Figure 5: Role of FAP in various tumor promoting mechanisms

Micro-angiogenesis

Aimes et al. observed Fibroblast activation protein production in human endothelial cells has function in relation to re-organization of micro-vasculature along-with morphological changes.[189]

This finding suggests that breast cancer cells that are positive for fibroblast activation protein do not proliferate in vitro, although after inoculating these cells into combined immunodeficient mice which leaded to increase in pace of tumor growth accompanied with a high degree of neo-vascularization. Esophageal cancer biopsy specimens evaluated histologically for expression of Fibroblast activation protein revealed that its intense expression was associated with a significantly higher density of microvessel.[190]

Fibroblast activation protein deletion or drug-based suppression of the same is linked to decreased microvessel density and slowed tumour growth, according to in vivo models of cancer cells from the lung and colon.[191].

Moreover, fibroblast activation protein was discovered to be produced by endothelial cells along the invasive front of ductal cancer, pointing to the protein's potential function in the neo-vascularization and invasion of the ECM.[192,193]. Additionally, there is a lot of Fibroblast activation protein expression in the stroma of glioblastomas that are growing abnormally.[194] Numerous findings have established that malignant tumour tissues, including those of multiple myeloma, gastric and breast cancer, express fibroblast activation protein on endothelial cells of microvascular origin.[195,196,197].

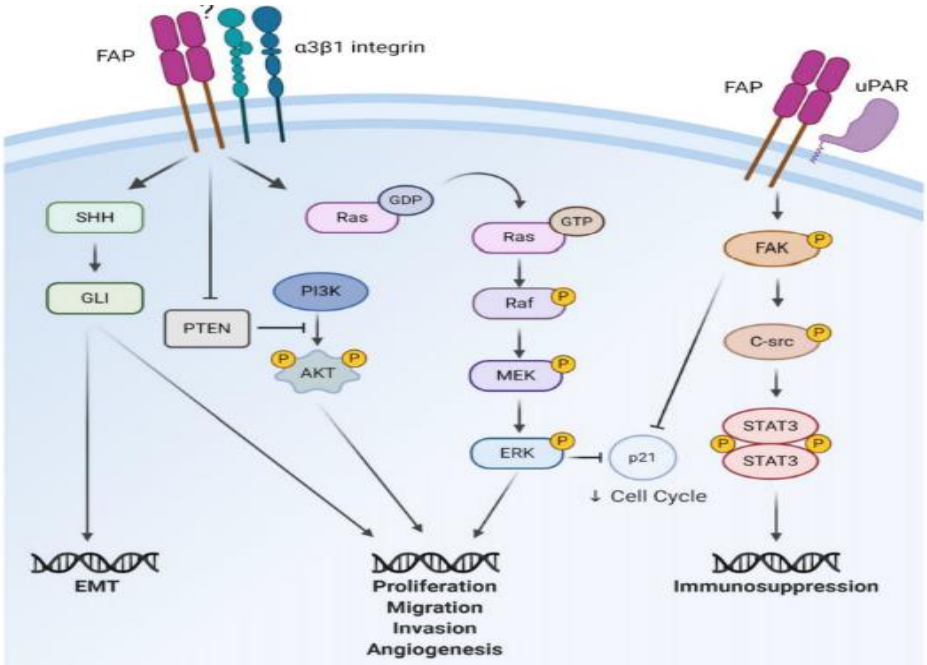


Figure 6: Potential signaling pathways affected by FAP expression that are responsible for the tumor promoting phenotypes associated with FAP expression.

"The role of fibroblast activation protein in health and malignancy" Allison A. Fitzgerald1 & Louis M. Weiner1 review art

Epithelial-to-Mesenchymal Transition/ EMT

Malignant epithelial cells can undergo an EMT transition, acquiring a mesenchymal phenotype that encourages enhanced motility and invasion necessary for LNM[158].

Fibroblast activation protein-targeting antibodies can be utilised to separate fibroblasts, according to studies.

During the EMT transition upon TGF- beta stimulation, several epithelial-derived cell lines also express fibroblast activation protein.[198].

Immunosuppression:

Immunomodulatory studies on Fibroblast activation protein positive Cancer Associated Fibroblasts demonstrated their contribution to priming an immunosuppressive TME by obstructing dendritic cell development and maturation pathways, preventing T cells from maturing into cytotoxic T cells[199], and inhibiting MHC antigen production. Cancer-associated fibroblasts that express the inflammatory gene C-X-C motif chemokine ligand2 with the greatest overexpression are known as fibroblast activation protein positive (CAP) fibroblasts.[200].

Fibroblast activation protein is present and positively associated with C-X-C motif chemokine ligand 12, mostly generated by activated fibroblasts, is a key mediator of local immunosuppression. Feig et al. [201] made use of this finding to note that the addition of inhibitors that target the C-X-C motif chemokine ligand 12 receptor, also known as C-X-C chemokine receptor type 4, led to a T-cell dependent reduction in tumour volume and an increased response to anti-PD-1 therapy.[202]

Kraman et al[203] used a transgenic mice model to establish the potential of fibroblast activation protein positive cancer associated fibroblasts to block the anti-tumor immune response and to confirm that FAP was expressed in various cluster of differentiation positive and negative CD45 cells. Fibroblast activation protein & its association with tumor invasion and lymph node metastasis highlighted by recent observational studies by

- According to H Wang et al., downregulating fibroblast activation protein in oral cancer inhibits cell growth and metastasis via disrupting the signalling pathways for phosphatase and tensin homolog, phosphoinositide 3-kinase, and Rat sarcoma viral oncogene homolog-mitogen-activated protein kinase. When fibroblast activation protein is turned down, metastatic cell motility and invasion are inhibited both in vitro and in vivo. When it is momentarily or permanently silenced, fibroblast activation protein expression is reduced in vitro and inhibits OSCC xenograft growth in vivo.

- N.Higashino et al. demonstrated that Fibroblast activation protein-positive fibroblasts promote tumor progression through secretion of C-X-C motif chemokine ligand 2 and interleukin-6 in Esophageal squamous cell carcinoma. Immunohistochemical investigation of ESCC tissues, showed increase in intensity of expression of two cancer Associated Fibroblasts markers—alpha smooth muscle actin (αSMA) and fibroblast activation protein (FAP)—in the tumor stroma which demonstrated a strong connection with LNM, advanced clinical stage, depth of tumour invasion, and worse prognosis.

- In their study, Wu et al. looked into the proteins that fibroblast activation protein targets and investigated the precise method by which this protein

encourages EMT in OSCC. They looked at proteins that interact with FAP, which promotes EMT of OSCC by non-enzymatically downregulating Dipeptidyl Peptidase 9. Fibroblast activation protein is highly expressed in fibroblasts associated with cancer and has been proven to have pro-tumorigenic activity. By using IP-MS, it was discovered that Dipeptidyl Peptidase 9 interacts intracellularly with Fibroblast Activation Protein. In OSCC tissue, Dipeptidyl peptidase 9 protein and mRNA levels were down-regulated. Un-favorable OSCC patient survival rates were connected with lower Dipeptidyl peptidase 9 expression. Dipeptidyl peptidase 9 knockdown increases OSCC cell growth.

- Li et al in their study on 121 resected specimens of ESCC and 10 adjacent normal tissue using IHC assessed the expression of Fibroblast Activation Protein in stromal fibroblasts close to neoplastic nests. There was no FAP-expression in the healthy controls. In 37% (45/121) of the ESCC patient tissues, there was evidence of stromal fibroblast activation protein expression. In ESCC patients, the expression of FAP- level is substantially connected with lymph node metastases but not with age, gender, or tumour site. In both univariable and multivariable analyses, stromal FAP-expression was strongly linked to poor survival. FAP- may have a key role in regulating ESCC lymph node metastasis and may offer a fresh therapeutic target for the disease..

Hence, with an overview of these above concepts, aim of our study is to find out the "**Expression of Fibroblast Activation Protein-Alpha in Oral Squamous Cell Carcinoma & its association with Lymph Node Metastasis**."

METHODOLOGY

Ethical Approval: Study was approved by Institutional review committee & ethical clearance was obtained with Registration number: 1462 (Annexure I). Basic training of ethics in human research was also undertaken.

Sample Size: Sample size included in our study is based on Convenience sample size. A total number of 90 Formalin Fixed Paraffin Embedded (FFPE) tissues blocks of histologically diagnosed radical neck dissection cases of Oral Squamous cell carcinoma were included in this study. All these cases were retrieved from the archives of Department of Oral Pathology and Microbiology KLE VK Institute of Dental Sciences.

Staining Procedure: Two sections of 3 µm thickness from formalin fixed paraffin embedded tissues were obtained. One section was placed on egg albumin coated slide for routine Hematoxylin and Eosin stain (Annexure V) to confirm the diagnosis and also to note the histological parameters included in our study. Another section was obtained on amino Propyl Tri-ethoxy Silane (APES) (Annexure IV), coated slide for immunohistochemical expression of FAPα. Immunohistochemical (IHC) staining of FAPα (MA543831) was performed using Invitrogen's FAP Monoclonal Antibody (C1) & Poly Excel HRP/DAB Two-step detection system (Cat# PEH002)

DEMOGRAPHIC DATA PARAMETERS:

Reporting of Radical Neck Dissection Cases in our department is according the guidelines by Royal college of Pathologists (United Kingdom). The demographic data of all the cases were retrieved from these records for Age, Sex, Site, Habit

history, Clinical diagnosis, Tumor Stage, Histological Grade, Invasion Status (Muscle Invasion, Neural Invasion & Lympho-vascular Invasion) and Nodal Metastasis were entered in an excel spread sheet.

EVALUATION OF HISTOPATHOLOGICAL PARAMETERS:

All the histopathological parameters included in our study is based on criteria's proposed by various authors. The invasion status of tumor cells was analyzed and recorded based on standard reporting of Royal college of Pathologists.

Whenever the tumor cells are invading into the muscle bundles was considered as Muscle invasion, tumor cells infiltrated within and around neural component was considered as Perineural Invasion (PNI) and Lympho-vascular invasion (LVI) was reported whenever the tumor cells are within the blood vessels and lymphatic channels.

Inflammatory response: Density and distribution of inflammatory cell in TME were analyzed in each case and was categorized as **Diffuse inflammation** (when the inflammatory cells are distributed diffusely around the tumor stromal component) and **Dense Inflammation** (whenever the inflammatory response is densely packed around the tumor stromal component)

Stromal Pattern: The pattern of distribution of collagen around the tumor component was considered for this parameter. Cases were categorized based on modification of Chatterjee et al either as **loosely arranged** collagen fibers or **hyalinized/ desmolytic** stroma surrounding the tumor islands.[55]

Tumor Budding: Tumor budding is analysed under x10 magnification at invasive tumor front as small tumor nests consisting of less than 5 cells as described by Wang et al.[137] After counting the number of tumor buds at invasive front, the intensity of tumor buds was graded according to Wang et al as **low intensity** consisting of <5 tumor buds and **high intensity** consisting ≥ 5 tumor buds at invasive tumor front.

Worst Pattern of Invasion (WPOI): All the lesional tumor slides were analyzed for the Pattern of Invasion (POI) at the tumor invasive front. Five types of POI was analyzed such as (i) POI 1- Pushing, well delineated infiltrating borders (ii) POI 2 - "Finger-like" pushing pattern (iii) POI 3 - Small groups or cords of infiltrating cells (n>15) (iv) POI 4 - Marked and widespread cellular dissociation in small groups of cells (n< 15) and POI 5 as tumor nodule which should be at least at a distance of one millimeter away from the tumor proper.[54] Each slide was analyzed for all 5 types of POI. Highest POI grade was considered as the Predominant POI (PPOI) which also denotes the WPOI for that particular case.[54]

Furthermore it was categorized according Kukreja P et al by combining WPOI 1,2,3 as **low aggressive variant** whereas WPOI 4& 5 were considered as **aggressive variant**.[59]

Depth of Invasion (DOI): According to Pentenero et al DOI is defined as the distance from the reconstructed mucosal surface to the deepest level of invasion.[7,59,138,139] It was measured by keeping the slide under lower magnification, with the help of marker pen the surface epithelium basement membrane was marked and subsequently deepest invasion of tumor was marked on the slide. Whenever there is ulcerated epithelium, adjacent epithelium basement membrane was considered as reference initial point. After marking down the surface and invasion end, slide was

removed and a line was drawn to join these two points. Then the slide was again kept under the lower magnification, a transparent scale was also kept on the slide under the microscope and DOI was measured in millimeters.[59,138]

1=Carcinoma in-situ/questionable invasion

2=Distinct invasion involving lamina propria

3=Invasion below the lamina propria adjacent to muscle, salivary gland and periosteum

4=Extensive and deep invasion replacing most of the stromal tissue in the jaw bone

IDENTIFICATION OF CANCER ASSOCIATED FIBROBLASTS:

Positive immunoreactivity to FAPα was defined when stromal cells with a spindle-shaped morphology presented at the interface with neoplastic epithelial islands exhibiting brown staining in the nucleus and/or cytoplasm at 10x and then at 40x magnification.

For the FAPα antibody positivity of various parameters included, the following scores were attributed according to staining intensity, the proportion of positive cells and location of the same.

S.no	Percentage of cells	Intensity	Location
0	**0-25% cells**	**Absent**	**Absent**
1	**26-50% cells**	**Mild**	**Cytoplasm**
2	**51-75%cells**	**Intense**	**Membrane**
3	**>75% cells**	**NA**	**Cytoplasm+ Membrane**
4	NA	**NA**	**Nucleus**
5	NA	**NA**	**Nucleus + Cytoplasm**

Principle of Immunostaining:

The PathnSitu's Poly Excel HRP/DAB two-step detection system is based on the principle of antigen/antibody reaction in tissues. Primary antibody of FAPα combines with its corresponding antigen in tissues after antigen retrieval. The identification of the antigen on the FFPE tissues is carried out using the above stated antibody. The antigen and antibody complex is visualized using an enzyme coupled (HRP/DAB) secondary antibody with specific binding to the primary antibody, this complex is visualized by the enzymatic activation of the chromogen resulting to a visible reaction production of the antigenic site. Each and every step involves precise time and optimal temperature and the results are interpreted using a light microscope by a qualified and trained pathologist.

Reagents Used:

a. Primary antibody

Specificity: FAP Monoclonal Antibody (C1)

i. Isotype: IgG2B

ii. Company: Invitrogen laboratories

b. Poly Excel HRP/DAB detection system Kit consists of:

i. PolyExcel Peroxidase Quencher (H_2O_2) (6 ml)

ii. PolyExcel Target Binder(6ml)

iii. PolyExcel PolyHRP (6ml)

iv. PolyExcel StunnDAB Chromogen (2 ml)

v. PolyExcel StunnDAB Substrate Buffer (10 ml)

c. Buffers:

i. Phosphate buffered saline (PBS): This was used as a wash buffer with pH ranging from 7.2-7.6. The preparation formula has been described in Annexure-VI.

ii. Citrate Buffer, pH 6.0 (Antigen Retrieval Solution): This was used for heat induced epitope retrieval (HIER) to unmask antigen binding sites in the tissues.

d. Graded Alcohol Solution (100%, 90%, 80%, 60%)

e. Xylene

f. Distilled water

g. Harris Hematoxylin

h. Mounting medium, DPX.

Other Equipments Used

1. APES coated glass slides
2. Staining trough
3. Humidifying chamber
4. Pressure cooker
5. Calibrated test tube
6. Plastic Pasteur pipette (to mix DAB chromogen & buffer)
7. Cover slips
8. Micropipettes
9. Semi automatic microtome (Leica RM 2145)
10. Slide warmer
11. Water bath
12. Multiviewer Microscope.

Immunohistochemical (IHC) Staining Protocol:

1. **Sectioning:** Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on APES coated slides. It was subjected to IHC using polyexcel HRP/DAB Two-step detection system (PEH2, Genepulse scientific, and Bangalore).
2. **Deparaffinization:** Slides were deparaffinized by keeping on a slide warmer at 60^{o}C for 1 hour and treated with two changes of xylene for 10 minutes each.
3. The slides were treated with one change each of 100% alcohol followed by graded alcohol 90%, 80%, 70% and 60% for 5 min each.
4. Slides were rinsed with distilled water.
5. **Heat induced:** Pressure cooker filled with Citrate Buffer, pH 6.0 was used as Epitope Retrieval method. After the three whistles were complete, the cooker was cooled till room temperature.
6. After the slides were cooled to room temperature they were washed in PBS (1&2) for 10 minutes each.

Immunohistochemical Staining:

1. Endogenous peroxidase activity was blocked by incubating the slides with PolyExcel **Peroxidase Quencher** for 15 minutes. Slides were washed with wash buffer PBS (1&2) for 5 minutes each.
2. Slides were incubated overnight with primary monoclonal antibody against FAPα for 18 hours in a humidifying chamber. After that slides were washed with wash buffer (PBS 1 & 2) for 5 minutes each.

3. **Polyexcel target Binder** enhances the binding capability of primary antibody to specific antigenic site by incubating slides for 15 minutes in humidifying chamber. This was followed by PBS 1 & 2 change for 5 minutes each.
4. **PolyExcel PolyHRP** added to promote Ag-Ab reaction and incubated for 20 minutes in humidifying chamber. This was followed by PBS 1 & 2 change for 5 minutes each.
5. Incubation with freshly prepared substrate/chromogen solution of DAB in provided buffer (by mixing 50 µl concentrated DAB in 1000 ul of substrate buffer for 10 slides) was done for 10 minutes to visualize antigen-antibody reaction. After that slides were dipped in distilled water to stop the reaction.
6. Slides were counterstained with Harris Hematoxylin for 1 minute.
7. Bluing was done in running tap water for 10 minutes.
8. After that slides were dehydrated and mounted with DPX.

Analysis of Immunoexpression of FAP-A antibody

Immunohistochemically stained sections with FAPα, were evaluated for percentage of cytoplasmic expression of the marker. Entire slide was scanned for analyzing the percentage of expression under lower magnification (x4) and also confirmed the expression under higher magnification (x10, x40). Each case was evaluated by two observers for the expression of FAPα in Epithelial & connective tissue cells.

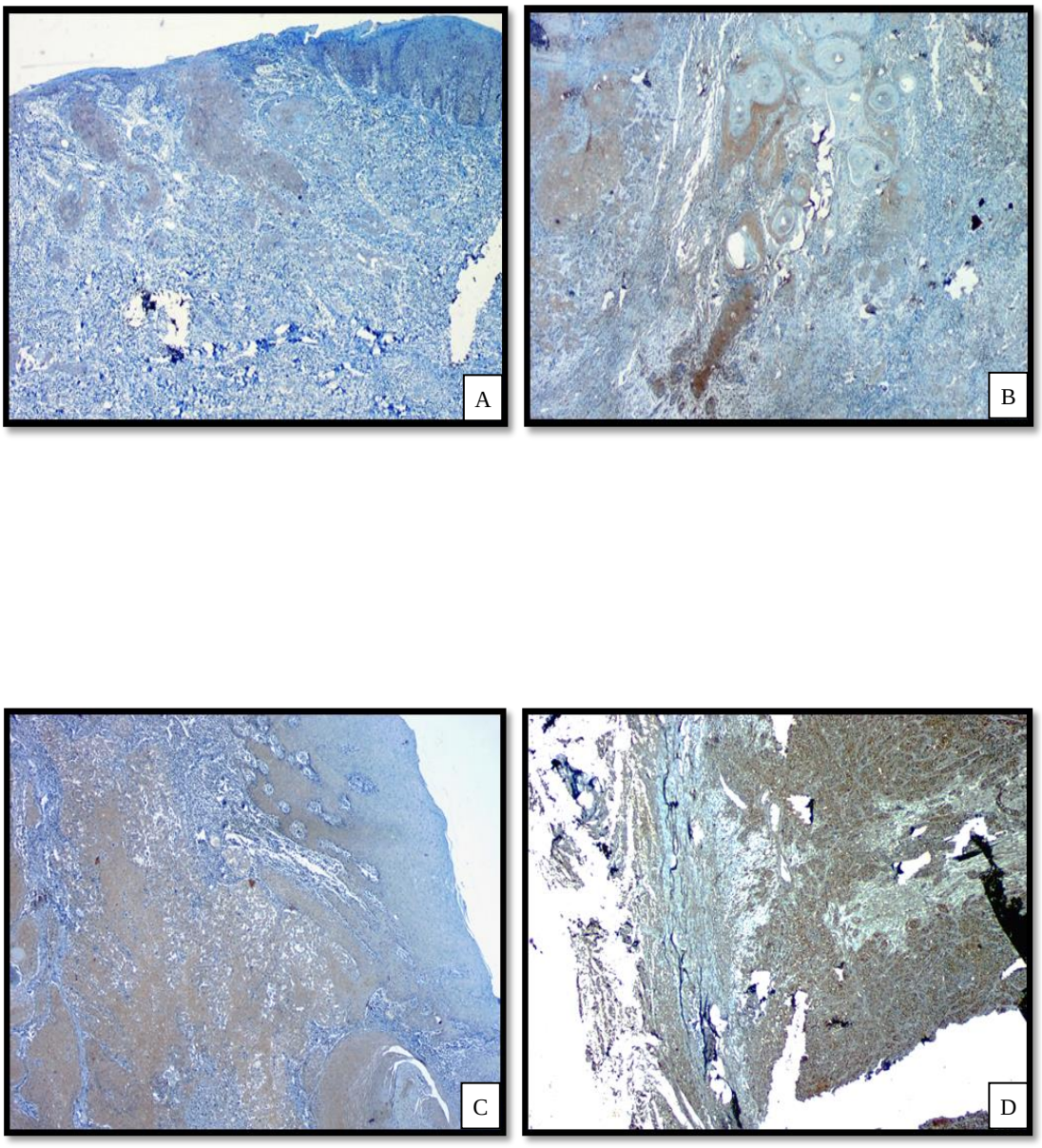
STATISTICAL ANALYSIS:

- The assessment of all histological parameters, FAPα expression in Cancer associated fibroblasts (CAF's) And Epithelial Tumor Cells were entered in a excel sheet. The data was then transferred to SPSS software (Version: 21.0) for application of statistical tests. At 95% confidence interval $p \leq 0.05$ was considered to be statistical significance.

- Frequency percentage was considered for all the demographic data parameters
- Association of all other histological parameters with FAPα expression was also done by chi-square test.

- Association of Lymph Node Metastasis with FAPα expression was done using Chi-square Test.

PHOTOMICROGRAPHS



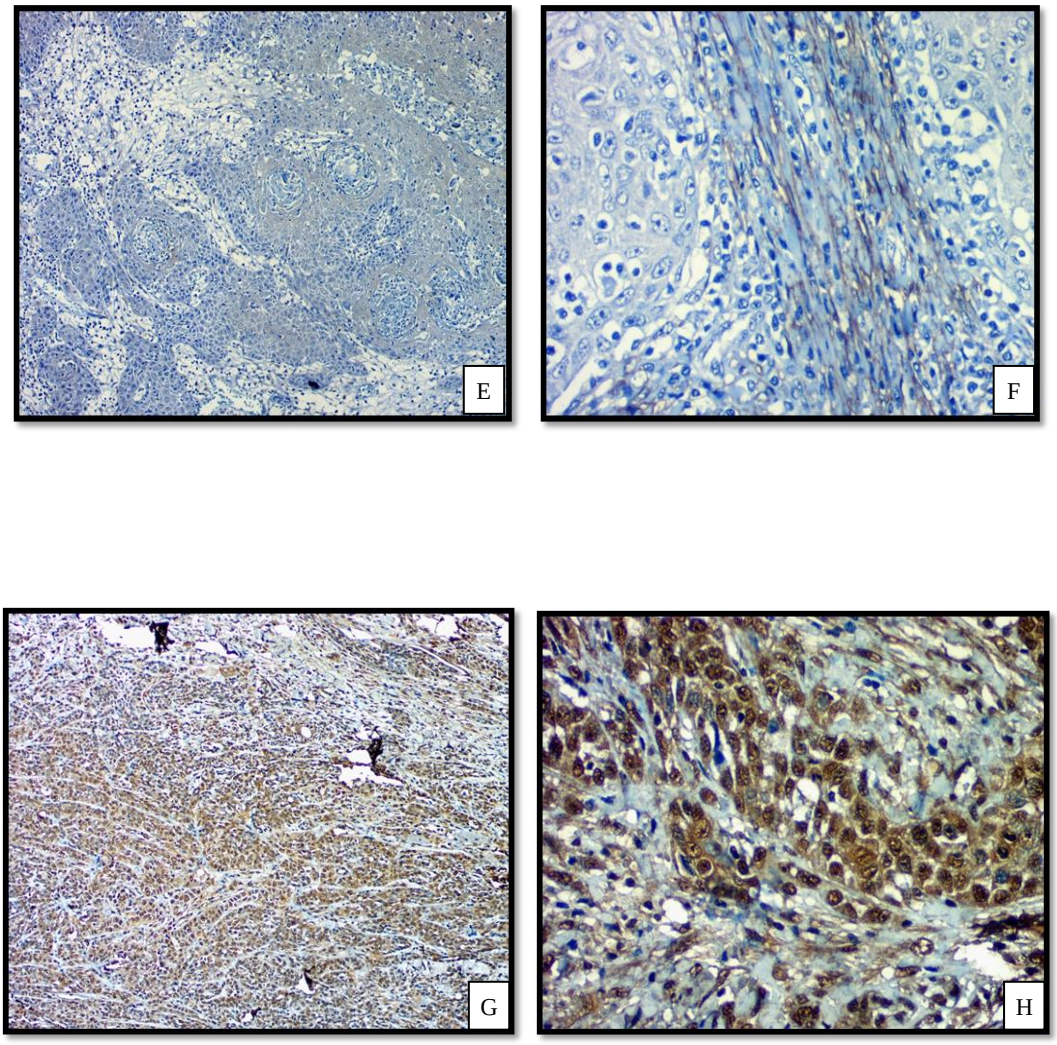
Photomicrograph 1:

A – Photomicrograph Showing 0% to 25% Positive Immuno-Expression of FAP- α Marker in OSCC

B – Photomicrograph Showing 26% to 50% Positive Immuno-Expression of FAP- α Marker in OSCC

C- Photomicrograph Showing 51% to 75% Positive Immuno-Expression of FAP- α Marker in OSCC

D– Photomicrograph Showing >75% Positive Immuno-Expression of FAP- α Marker in OSCC



Photomicrograph 2:

E – Photomicrograph Showing Mild Positive Immuno-Expression of FAP- α Marker in OSCC: Labelling Index 1(Ax10 &B x40)

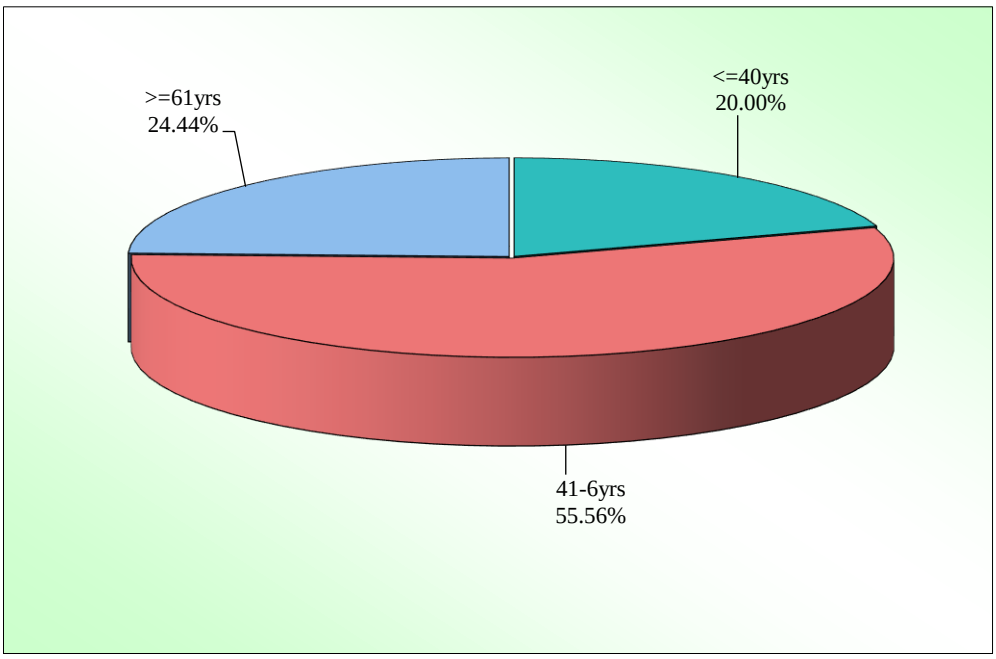
F – Photomicrograph Showing Mild Positive Immuno-Expression of FAP- α Marker in OSCC & Intense positivity in CAF

G- Photomicrograph Showing Mild to Intense Positive Immuno-Expression of FAP- α Marker in OSCC & CAF

H – Photomicrograph Showing Intense Positive Immuno-Expression of FAP- α Marker in OSCC cells & CAF's

RESULTS

Graph 1: Pie Chart depicting Age wise distribution of Demographic Data in Percentage (Total = 90)



Graph 2: Pie Chart depicting gender wise distribution of Demographic Data in Percentage (Total = 90)

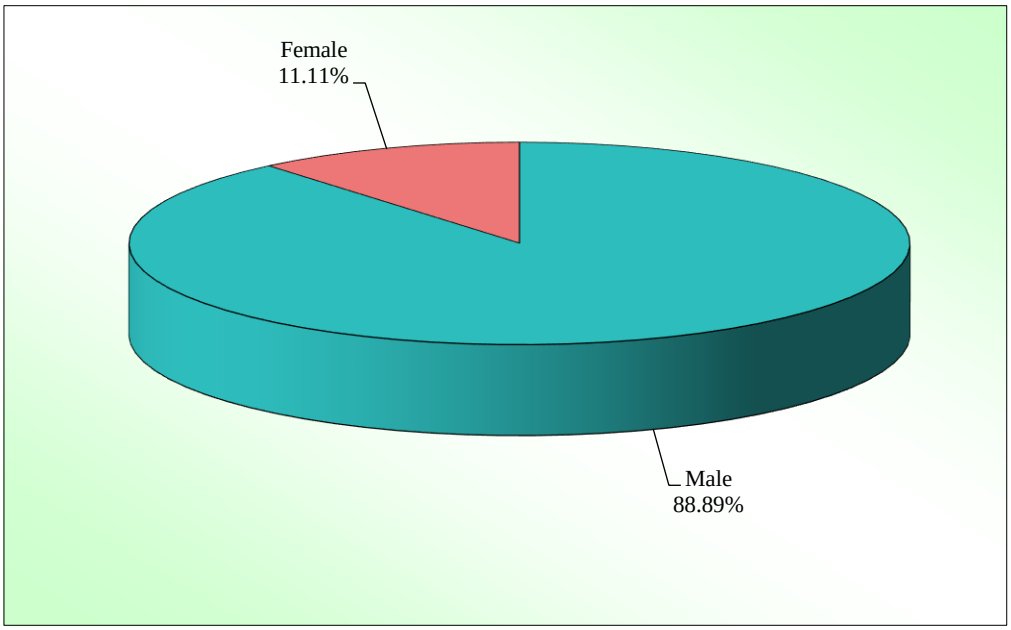


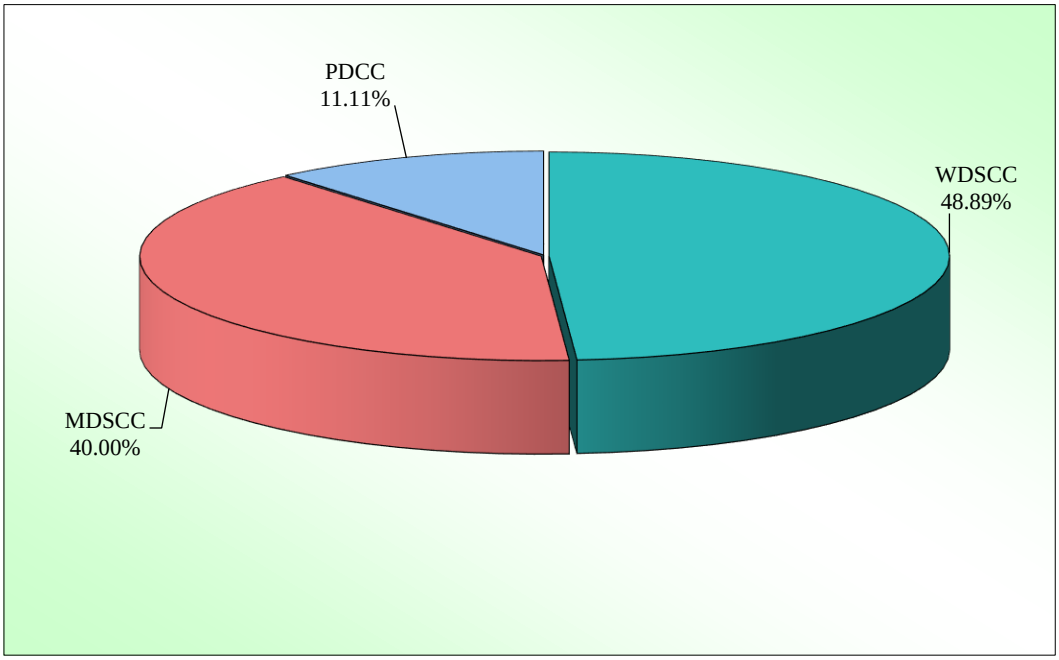
Table 1: Site wise distribution for Oral cancer

Site	Number	Percentage
Buccal mucosa	54	60.00
Tongue	12	13.33
Lip	2	2.22
Alveolus	5	5.56
Palate	1	1.11
Gingivo-buccal sulcus	7	7.78
RMT	9	10.00
Total	90	100.00

Description: Out of total number of 90 cases included in our study, we found that cases with less than equal to 40 years of age were 20% with 55.56% belonging to 41-60 years of age group and more than or equal to 61 years of age were 24.4%. In our study majority of the subjects were male individuals comprising 88.89% of study sample, whereas females were 11.11% of study sample. On considering the type of tobacco habit we found that 62.22% of our cases included had tobacco habit in the form of chewing. On considering the site predilection in our cases, we found majority of the cases belonged to Buccal mucosa and Tongue followed by RMT & GBS region. Whereas, only 10% of sample belonged to other sites of Oral cavity like, Labial mucosa, Palate, Floor of Mouth.

Inference: Demographic data showed predominance of individuals belonging to 41 to 60 years of age with predominantly male participants. All our cases had chewing type of tobacco with buccal mucosa as the predominant site.

Graph 3: Pie Chart depicting Grade wise distribution of Demographic Data in Percentage (Total = 90)

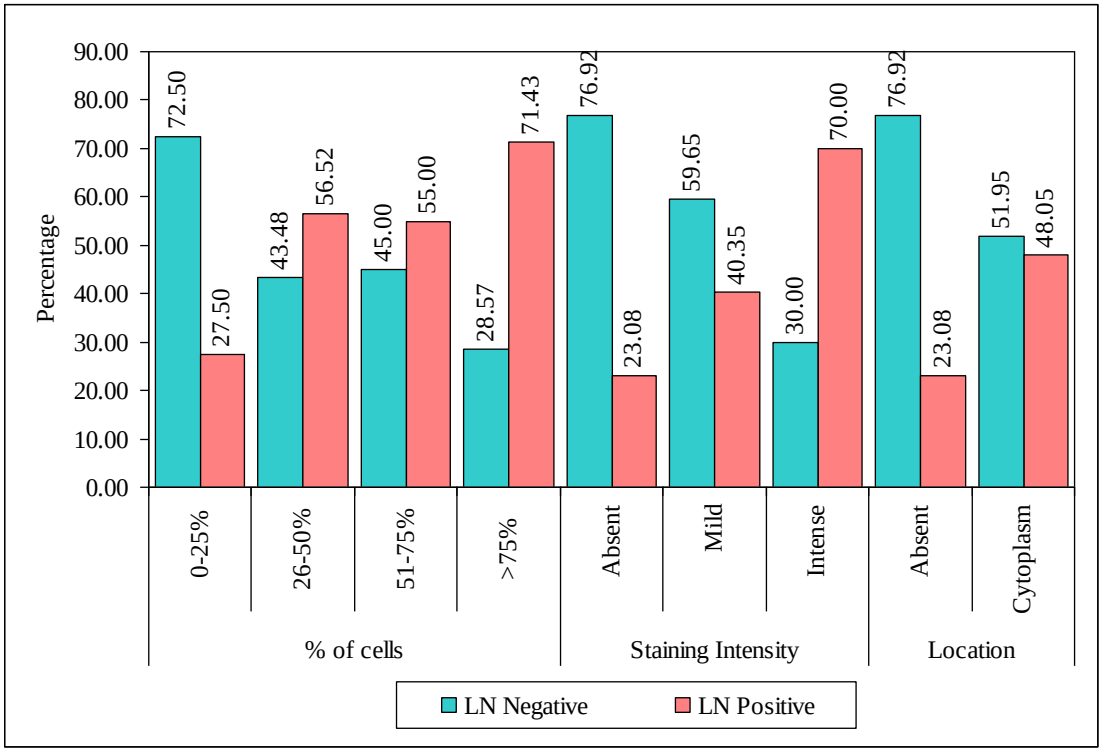


Description: Out of total 90 cases included in our study, on considering the histological grade majority our cases 48.89% were WDSCC followed by 40.0% of MDSCC and only 11.11% of our cases were PDSCC. On considering the Lymph node metastasis, we found 53.3% (n=48) negative nodal status and 46.6% (n=42) showed positive nodal status. On observing the tumor invasion status we found 34.4% (n=31) and 41.1% (n=37) of our cases showed positive for PNI and LVI respectively. On analysing the intensity of presence of tumor budding status, we found 38.8% (n=35) of our cases were of Less intense and 61.1% (n=55) of our cases showed high intense. Considering WPOI (1, 2, 3) our cases belonging to less aggressive were 84.44% (n=76) and WPOI (4&5) considering to be aggressive were 15.55% (n=14) of our cases. On assessing DOI measurement, we found majority of the cases belonged to group 3 (68%), grade 2 (22.22%) followed by grade 4 & 1 (3.3% & 1.1%) respectively.

Description: Demographic data parameters were subjected to Chi-square test to find the association of these parameters with FAPα expression in both epithelial cells and Cancer associated Fibroblasts considering p<0.005 as statistical significance with 95% confidence interval. We didn't find any significant association between the demographic parameters except for age groups which showed statistical significant association with intensity of FAPα expression.

Inference: Statistical significant association was observed between intensity of FAPα expression in CAFs and age groups using Chi-square test (x^2= 12.3610, p < .05). No other demographic parameters showed any significant association.

Graph 4 : Comparison of LN status with percentage of cells, stating intensity and location of Cancer Associated Fibroblasts (CAFs)



Description: There is significant association between, lymph-node metastasis and expression of FAPα in cancer associated fibroblasts. We found significant association between lymph node metastasis and presence of increased FAPα positive CAF in the connective tissue stroma ($\chi 2 = 8.9770$, $p < .05$). We also noted that with increase in staining intensity of CAF by FAPα significant association was noted with Lymph Node Metastasis, with ($x^2 = 8.0810$, $p < .05$).

Inference:

Lymph node Metastasis showed statistically significant association with both increase in number of FAP-α positive CAF's ($x^2 = 8.9770$, $p < .05$) & increase in staining intensity of FAP-α in CAF's ($x^2 = 8.0810$, $p < .05$) using Chi-square test.

Table 2: Association between Lympho-vascular Invasion (LVI) with percentage of cells, stating intensity and location of epithelial cells, C.T. Component and Fibroblasts

Factors	Absent	%	Present	%	Total	%	Chi-square	p-value
Epithelial cells								
% of cells								
0-25%	13	72.22	5	27.78	18	20.00	6.1260	0.1060
26-50%	13	54.17	11	45.83	24	26.67		
51-75%	13	76.47	4	23.53	17	18.89		
>75%	14	45.16	17	54.84	31	34.44		
Staining Intensity								
Absent	4	100.00	0	0.00	4	4.44	3.9970	0.1360
Mild	25	52.08	23	47.92	48	53.33		
Intense	24	63.16	14	36.84	38	42.22		
Location								
Absent	4	100.00	0	0.00	4	4.44	4.6940	0.0960
Cytoplasm	48	58.54	34	41.46	82	91.11		
Nucleus	1	25.00	3	75.00	4	4.44		
C.T. Component								
% of cells								
0-25%	20	64.52	7	22.58	31	34.44	6.3860	0.0940
26-50%	19	73.08	7	26.92	26	28.89		
51-75%	6	46.15	12	92.31	13	14.44		
>75%	8	40.00	37	185.00	20	22.22		

Staining Intensity								
Absent	8	80.00	2	20.00	10	11.11	2.1360	0.3440
Mild	37	56.92	28	43.08	65	72.22		
Intense	8	53.33	7	46.67	15	16.67		
Location								
Absent	9	81.82	2	18.18	11	12.22	2.7210	0.0990
Cytoplasm	44	55.70	35	44.30	79	87.78		
Fibroblasts								
% of cells								
0-25%	27	67.50	13	32.50	40	44.44	11.0070	0.0120*
26-50%	17	73.91	6	26.09	23	25.56		
51-75%	6	30.00	14	70.00	20	22.22		
>75%	3	42.86	4	57.14	7	7.78		
Staining Intensity								
Absent	10	76.92	3	23.08	13	14.44	2.0490	0.3590
Mild	32	56.14	25	43.86	57	63.33		
Intense	11	55.00	9	45.00	20	22.22		
Location								
Absent	10	76.92	3	23.08	13	14.44	2.0410	0.1530
Cytoplasm	43	55.84	34	44.16	77	85.56		
Total	53	58.89	37	41.11	90	100.00		

Table 3: Association between Tumor budding with percentage of cells, stating intensity and location of epithelial cells, C.T. Component and Fibroblasts

Factors	Low	%	High	%	Total	%	Chi-square	p-value
Epithelial cells								
% of cells								
0-25%	13	72.22	5	27.78	18	20.00	12.7940	0.0050*
26-50%	6	25.00	18	75.00	24	26.67		
51-75%	6	35.29	11	64.71	17	18.89		
>75%	8	25.81	23	74.19	31	34.44		
Staining Intensity								
Absent	4	100.00	0	0.00	4	4.44	7.3650	0.0250*
Mild	17	35.42	31	64.58	48	53.33		
Intense	12	31.58	26	68.42	38	42.22		
Location								
Absent	4	100.00	0	0.00	4	4.44	9.2850	0.0100*
Cytoplasm	29	35.37	53	64.63	82	91.11		
Nucleus	0	0.00	4	100.00	4	4.44		
C.T. Component								
% of cells								
0-25%	15	48.39	15	48.39	31	34.44	8.3320	0.0400*
26-50%	11	42.31	8	30.77	26	28.89		
51-75%	5	38.46	18	138.46	13	14.44		
>75%	2	10.00	57	285.00	20	22.22		
Staining Intensity								
Absent	4	40.00	6	60.00	10	11.11	0.7840	0.6760
Mild	25	38.46	40	61.54	65	72.22		
Intense	4	26.67	11	73.33	15	16.67		
Location								
Absent	5	45.45	6	54.55	11	12.22	0.4170	0.5190
Cytoplasm	28	35.44	51	64.56	79	87.78		

Fibroblasts								
% of cells								
0-25%	20	50.00	20	50.00	40	44.44	9.5670	0.0230*
26-50%	9	39.13	14	60.87	23	25.56		
51-75%	4	20.00	16	80.00	20	22.22		
>75%	0	0.00	7	100.00	7	7.78		
Staining Intensity								
Absent	7	53.85	6	46.15	13	14.44	4.1360	0.1260
Mild	22	38.60	35	61.40	57	63.33		
Intense	4	20.00	16	80.00	20	22.22		
Location								
Absent	7	53.85	6	46.15	13	14.44	1.9310	0.1650
Cytoplasm	26	33.77	51	66.23	77	85.56		
Total	33	36.67	57	63.33	90	100.00		

Description: There is no significant association noted between histological tumor grade, DOI, POI, PNI, stromal type and inflammatory response with respect to expression of FAP-α in both CAF & Epithelial cancer cells. However only two variables namely TB and LVI were significantly associated with FAP-α expression on CAF. We noted that increase in tumor budding activity considering to be high intense showed significant association, both with increase in number of FAP-α positive epithelial cells and number of Cancer associated fibroblast in the connective tissue stroma (>75% cells) with $x^2 = 12.7940, p < 0.05$ and $x^2 = 9.5670, p < 0.05$, respectively. Considering LVI, there was significant association noted with increase in number of FAP-α positive CAF and presence of Lympho-Vascular Invasion with $x^2 = 11.0070, p < .05$.

Inference:

Among various Histopathological parameter Tumor Budding showed statistically significant association with expression of FAP-α in both Epithelial cells (x^2=12.7940, $p < .05$) and CAF (x^2= 49.5670, $p < .05$) using Chi-square test.

Significant association was also noted with increase in number of FAP-α positive CAF and presence of Lympho-Vascular Invasion with (x^2= 11.0070, $p < .05$) using Chi-square test.

Table 4: Association between Margins with percentage of cells, stating intensity and location of epithelial cells, C.T. Component and Fibroblasts

Factors	Absent	%	Present	%	Total	%	Chi-square	p-value
Epithelial cells								
% of cells								
0-25%	15	83.33	3	16.67	18	20.00	5.9290	0.1150
26-50%	19	79.17	5	20.83	24	26.67		
51-75%	11	64.71	6	35.29	17	18.89		
>75%	17	54.84	14	45.16	31	34.44		
Staining Intensity								
Absent	4	100.00	0	0.00	4	4.44	3.1280	0.2090
Mild	30	62.50	18	37.50	48	53.33		
Intense	28	73.68	10	26.32	38	42.22		
Location								
Absent	4	100.00	0	0.00	4	4.44	5.4160	0.0670
Cytoplasm	57	69.51	25	30.49	82	91.11		
Nucleus	1	25.00	3	75.00	4	4.44		
C.T. Component								
% of cells								
0-25%	26	83.87	6	19.35	31	34.44	10.4940	0.0150*
26-50%	20	76.92	7	26.92	26	28.89		
51-75%	6	46.15	10	76.92	13	14.44		
>75%	10	50.00	28	140.00	20	22.22		
Staining Intensity								
Absent	8	80.00	2	20.00	10	11.11	2.3770	0.3050
Mild	46	70.77	19	29.23	65	72.22		
Intense	8	53.33	7	46.67	15	16.67		
Location								
Absent	9	81.82	2	18.18	11	12.22	0.9770	0.3230
Cytoplasm	53	67.09	26	32.91	79	87.78		

Fibroblasts								
% of cells								
0-25%	34	85.00	6	15.00	40	44.44	18.1830	0.0001*
26-50%	17	73.91	6	26.09	23	25.56		
51-75%	10	50.00	10	50.00	20	22.22		
>75%	1	14.29	6	85.71	7	7.78		
Staining Intensity								
Absent	9	69.23	4	30.77	13	14.44	7.1600	0.0280*
Mild	44	77.19	13	22.81	57	63.33		
Intense	9	45.00	11	55.00	20	22.22		
Location								
Absent	9	69.23	4	30.77	13	14.44	0.0010	0.9770
Cytoplasm	53	68.83	24	31.17	77	85.56		
Total	62	68.89	28	31.11	90	100.00		

Description: There is significant association between, positive & negative surgical margins and expression of FAP-α positive cancer associated fibroblasts. 66.66%(n=60) of the cases had negative/free margins with 33.33%(n=30) cases having positive margins. We noted that with presence of positive margins, an increase in both number & staining of FAP-α in CAF showed significant association with(x^2= 18.1830, $p < .05$) & (x^2= 7.1600, $p < .05$) respectively.

Inference: Histopathological parameters like Status of surgical margins showed statistical significant association with increase in both number & staining intensity of FAP-α using Chi-square test. (x^2= 18.1830, $p < .05$) & (x^2= 7.1600, $p < .05$)

DISCUSSION

Cancer is a collective terminology that refers to large group of diseases which has the propensity to develop in any part of the body. Lately, in 2021 World Health Organization (WHO) proposed cancer as second most common cause for death worldwide and among these deaths, tobacco habit is considered as one of the main causative agents.[204] In India, prevalence of Oral cavity cancer is predicted to increase by 26%, by 2030. [205] Oral cancer is the sixth most prevalent type of cancer in the world, with India accounting for roughly a third of the total burden and having the second-highest number of cases. With potentially malignant diseases also acknowledged as a clinically apparent stage of oral cancer, OSCC predominates all cases of oral cancer. Consumption of tobacco in the form of Smoking betel nut, chewing betel quid, drinking too much alcohol, having bad oral hygiene, and having persistent viral illnesses are some of the risk factors for the cause of oral cancer. Lack of self- knowledge and awareness, with variations in exposure to various environment factors, with behavioural risk factors indicate a wide variation in the global incidence and increases the mortality rate.[206] OSCC persists as the most commonly occurring mouth neoplasms as compared to other oral malignancies.[207] Globocan 2020 survey stated lip and oral cavity cancer as the most prevalent cancer in India.[208] Though the etiology of OSCC is multifactorial, in India tobacco is considered as the main causative agent. Among various types of tobacco habit, chewing/smokeless tobacco is considered to be the most prevalent habit for the development of OSCC in our country.[209]

Despite of numerous researches conducted in OSCC, the prognostic status of OSCC is still considered to be low. This can be mainly attributed to delay in seeking treatment, whereby the tumor must have already progressed into an advanced stage and also due to the poor dietary habits. Moreover, the incidence of OSCC in young individuals is also noted to be high.[210] Therefore it is of utmost importance to diagnose OSCC at an early stage and also to assess its prognostic status. It has been found to be challenging for onco-biologists, clinicians and researchers to identify the factors which will aid in predicting the prognosis of oral cancer and its metastatic potential to regional & distant structures.

The principal parameters which help the pathologists to predict the prognostic status of the tumor is always credited to the histopathological parameters that can be observed under H&E-stained sections. To mention few are PNI, LVI, TB, DOI, Lymph-node metastasis, extra-nodal extension etc.[211,212,213,214,215] All the above-mentioned parameters have already established their role in determining the prognosis of OSCC.

The connective tissue stroma's predominant cell population, fibroblasts also serve as a key source of the ECM and soluble substances that make up the acellular tissue microenvironment.

Fibroblasts through (i) direct cell interaction (ii) paracrine signalling via soluble factors and (iii) preserving matrix integrity, perform a variety of suppressive actions against cancer-causing and -metastatic cells.

Exhaustion of these tumor-suppressing abilities, carried out by fibroblasts, is a natural step in tumour development. Normal fibroblasts that have been transformed by

tumour cells into cancer-associated fibroblasts release a variety of pro-tumorigenic signals. These signals are caused by a disruption of the normal tissue architecture, which creates the ideal environment for the proliferation of cancer cells in the connective tissue stroma, promoting tumour growth and metastasis.

Increasing literature-based evidence points to Cancer associated fibroblasts manifesting a major role in the growth and metastasis of many tumor types[216,217,218]. So to understand the mechanisms of such interactions between Cancer associated fibroblasts and tumor cells may contribute to the future development of cancer therapy.

There are several hypotheses stated in regard to formation of **CAF & their role in tumor initiation and progression rate** with multiple observations from various cell culture and FFPE studied tissues highlighting their contribution in the process of tumor progression and metastsis.

Using both an in vivo tissue recombination system and an in vitro co-culture system, Olumi, A.F. et al.[222] investigation on CAF roles on several mouse and human cell experimental models on fibroblasts linked with carcinomas boost tumour progression of initial non-tumorigenic epithelial cells..

Human carcinoma-associated fibroblasts significantly accelerate proliferation and change the histology of epithelial cells when cultivated with activated human epithelial cells. It was determined that carcinoma-associated fibroblasts stimulate tumour advancement of an activated epithelial cell since this effect was not seen when cultured normal fibroblasts with the same epithelial cells under the same experimental conditions.

In their cell culture investigation on CAFs derived from prostate cancer patients, Hayward, S.W., Wang, et al. found that CAFs trigger EMT and immortalization capacities as well as change the quiescent cells' from non-tumorigenic characteristics into more tumorigenic ones.[221]

Kojima, Y & Acar.A et al[219] in their cell culture study on Cancer associated fibroblasts & myofibroblast-rich cell populations, extracted from human cancers concluded that Cancer associated fibroblasts & myofibroblast-rich cell populations maintain an ability to promote tumorigenesis.

Since epithelial cells can change into a 'fibroblast' like phenotype through undergoing epithelial-mesenchymal transititon in inflammatory processes and cancer, Kalluri R et al. claimed that epithelial cells can be a substitute source of fibroblast-like cells. Tumor-promoting by creating Transforming Growth Factor and Stroma Derived Factor1, cancer-associated fibroblasts and myofibroblasts can develop from pre-existing stromal fibroblasts. autocrine signalling pathway during the development of tumours.[220]

Cancer cells eventually colonise tissue in a distant organ to create a metastatic habitat. By enlisting stromal cells at their premetastatic site, they may channel to the target tissue in advance to accomplish this.

Mezawa, Y, Orimo, A et al. in their study observed that fibroblasts are increasingly converted into tumor-promoting Cancer associated fibroblasts myofibroblasts through the establishment of cross-communicating Transforming growth factorβ and Stroma derived factor autocrine signalling during tumorigenesis[223]. Infiltrating cancer stem cells can also recruit fibroblasts from lungs

to overexpress periostin, which in turn stimulates Wingless related integration site signalling in tumor cells and enhances their colonizing efficiency[224]

Aside from remodelling fibrillar ECM structures, cancer-associated fibroblasts release matrix-cellular proteins that act as a bridge between the stromal Extra-cellular matrix and the cancer cells. They also change the cancer cells' diverse signalling pathways, which promote invasion and metastasis.[225] 1 of those protein is Fibroblast activation protein alpha whose overexpression is noted in many tumor tissues, including breast[134-137], colorectal[138-139], pancreatic[141-144], lung[145-147], brain[148-150], intrahepatic bile duct[151], and ovarian[152-155]cancers.

Fibroblast Activation Protein expression is often low or undetectable in tissues that are systemically healthy, but it is overexpressed in several malignancies. Numerous malignancies, including those of the breast, colon, pancreatic, ovarian, lung, and bladder, have been discovered to overexpress fibroblast activation protein.

As fibroblast activation protein- is typically highly expressed in the stroma of different malignancies, it has evolved into a recognised marker of fibroblasts that are connected to cancer.

The impact of Fibroblast Activation Protein-on cell proliferation, migration, and invasion—all of which encourage tumour growth—may be the finding in the literature that is most consistently seen. Fibroblast Activation Protein- has been shown to encourage the invasion of cells from the endothelium, melanomas, ovarian and oral malignancies, and connective tissue components like fibroblasts.[226-230]

In their study, Wu et al. looked into the proteins that Fibroblast Activation Protein targets and investigated the precise mechanism by which Fibroblast

Activation Protein promotes epithelial mesenchymal transition in cancers. They came to the conclusion that FAP promotes EMT of oral cancers by non-enzymatically downregulating DPP9.

Li et al in their study on 121 resected specimens of Esophageal Squamous Cell Carcinoma and 10 adjacent normal tissue using immunohistochemistry evaluated Fibroblast Activation Protein-α expression in the stromal fibroblasts adjacent to neoplastic nests and co-related expression of FAP-α with LNM.

Regional lymph node metastasis represents a poor prognostic factor in head and neck squamous cell carcinoma. The presence of just 1 metastatic lymph node confers patients to an advanced disease stage and has been shown to confer up to a 50% decrease in overall survival.[231]

Nodal burden which are metastatic are central predictors of mortality in patients with oral cancer, with each additional metastatic lymph node contributing to escalated risk of mortality.

Fibroblast Activation Protein-α has also been considered as a potential immunotherapeutic target[232]Clinically effective FAP targeting strategies include blocking Fibroblast Activation Protein-proteinase activity with small molecules or antibodies, cleaving oncologic drugs attached to peptides targeted to FAP through Fibroblast Activation Protein-proteinase activity, vaccination against FAP, and, most recently, Fibroblast Activation Protein-Chimeric Antigen Receptor T cells. These developments could pave the way for more effective ways to explain patient prognostic characteristics, customise clinical trial designs, and eventually advance clinical design for cancer patients' treatment plans.

Fibroblast activation protein has been studied in various carcinomas but has not been extensively studied in OSCC tissues.

By altering signalling pathways in oral cancer, H. Wang et al. showed that downregulating fibroblast activation protein inhibits cell growth and metastasis. Fibroblast Activation is reduced. In cell cultures and FFPE tissues, protein reduces cell invasion and migration. either permanently diminished or briefly muted Expression of the fibroblast activation protein inhibits cell proliferation in vitro and reduces the growth of cancer cell xenografts in vivo. Reduced Fibroblast Activation Protein prevents cell invasion and migration both in vitro and in vivo.

These findings imply that cancer-associated fibroblasts are not only necessary for the initiation of tumours but also for the progression of an already established growth. They also act as a bridge between the cancer cells and the stromal extracellular matrix and modify various cancer cell signalling cascades, which facilitate metastasising and invasive capabilities.

Therefore, with these above-mentioned concepts the aim of our study was to find an association between expression of Fibroblast Activation Protein-alpha by Cancer associated fibroblasts and cancer cells with Lymph node metastasis and other prognostic histopathological parameters.

Demographic data can aid in determining the age predilection, habit and site involved in the study. Most of the literatures depicts an association of higher age range with poorer prognosis in malignancies including OSCC.[233-235] This can also be related to increase in co-morbidities with age.[236] On analyzing the frequency percentage of the demographic data of patients included in this study, out of 90 RND we found that

cases with less than equal to 40 years of age were 20% and more than or equal to 61 years of age were 24.4%. In our study majority of the subjects were of male individuals comprising of 88.89% of study sample, whereas females were 11.11% of study sample. On considering the type of tobacco habit we found that 62.22% of our cases included had tobacco habit in the form of chewing. On considering the site predilection in our cases, we found majority of the cases belonged to Buccal mucosa and Tongue followed by RMT & GBS region. Whereas, only 10% of sample belonged to other sites of Oral cavity like, Labial mucosa, Palate, Floor of Mouth. (Table 1)

On noting down site predilection, predominant site noted in this study is buccal mucosa and Gingivo-Buccal sulcus which together constitutes 67.78% followed by other sites such as floor of mouth, tongue, palate, lip etc (Table1). Buccal mucosa outnumbered undoubtedly in our sample which could be due to the most common type of tobacco habit prevalent in this region in the form of chewing along with the practice of keeping tobacco in the form of Quid in BM vestibule for longer durations (Table 1). This habitual placement can cause continuous friction as well as irritation to the oral mucosa followed by ulcer formation. Acharya et al, in their study which is done among similar population have also noticed a site predominancy for buccal mucosa in development of OSCC.[237] Though the association of site and expression of **FAPα** was found to be insignificant, we found significant association between age groups and intensity of **FAPα expression in CAF**.

On considering the Tumor grade, we could not find associate any significance between **tumor grades** and **Fibroblast Activation Protein α expression in both Cancer cells and Cancer associated fibroblasts**. On analyzing tumor cells for **FAP-**

α expression we noted that 20% of the cases show 0-25% positivity, 26% of the cases showed FAP-α expression in 26-50% of the tumor cells, 18.88% of the cases showed positivity in 51-75% of the cells & 33.33% of the cases showed positivity for FAP-α in >75% of the tumor cells.

In OSCC it is well proven that **lymph-node metastasis** is considered to have poor prognosis with treatment of OSCC also based on positive lymph node status. In our study out of 90 cases analyzed, 25 cases showed positive for lymph nodes metastasis. We found statistically significant association of **FAPα expressing Epithelial cells & CAFs** with lymph node metastasis. (Graph 4) The results are in accordance to those observations made by Li et al in their study on 121 resected specimens of Esophageal Squamous Cell Carcinoma using Fibroblast Activation Protein-α expression in the stromal fibroblasts adjacent to neoplastic nests in which they concluded that stromal Fibroblast Activation Protein -α expression was significantly associated with poor survival in both uni and multivariable analysis and Fibroblast Activation Protein -α may be an important regulator of LMN in ESCC.

As mentioned earlier some of the histopathological parameters are considered as prognostic indicators during routine reporting of OSCC, hence we made an attempt to find an association of FAP-α expression in CAF's and cancer cells with histopathological parameters. One such parameter which recently gained attention is **Tumor budding activity**. Tumor budding (TB) mainly represents the invasive characteristic of the tumor cells, where the cells are displaced in small groups from the main tumor mass.[212] This dissemination of cancer cells from the main tumor mass occurs mainly due to Epithelial-mesenchymal transition. Whereby, tumor cells loses epithelial cell characteristics and becomes highly motile mesenchymal like cells

contributing to the invasive nature followed by metastasis of the tumor.[212] It is also proved that TB activity has significant association with LNM and OS status in Oral Cancer.[238] Additionally, high budding activity is also associated with high proliferative ability of the cells.[239] In our study we observed that high intense tumor budding activity had a significant association with increase in presence of **FAPα** expressing Epithelial cells and Cancer associated fibroblasts with an increase in staining intensity noted for epithelial cells(Table 3).

Other histopathological parameters such as **Worst patten of invasion (WPOI), Depth of Invasion measurement (DOI)** did not show statistically significant association of increase of **FAPα expressing Epithelial cells & Cancer associated fibroblasts** with the increase in grades of POI & DOI.

On analyzing the other features of TME such as **stromal pattern and inflammatory response,** we could not find statistical significant association between expression of **FAPα in Cancer associated fibroblasts and Epithelial cancer cells**. Though there are various studies in the literature showing association of FAP**α expression with decrease in inflammatory response to tumor.**

As all of these parameters like LNM, TB and LVI are associated with increase in invasion and metastatic potential of the tumor cells. We observed an increase in presence of **FAPα expressing Epithelial cells & CAF's and its significant association to Tumor budding and Lympho-vascular invasion (LVI) which could point to the invasiveness capabilities of CAF and their role in tumor progression**.

Moreover, we found significant association between surgical margins and FAP-α expression in CAF's. We noted that with presence of positive margins, an

increase in both number & staining of FAP-α in CAF showed significant association. (Table 4)

Since an adult with a healthy immune system does not have excessive Fibroblast Activation Protein expression in other tissues or in the TME. Additionally, it has been noted that in oral SCC cells, Fibroblast Activation Protein-α knockdown inhibited proliferation, migration, and invasion by inactivating a number of signalling pathways.[240]

Hence our study is the first of its kind to show significant association of Fibroblast Activation Protein-α α expression in CAFs with LNM in Oral Squamous Cell Carcinoma.

Though there are several mechanisms for formation of cancer associated fibroblasts, we would like to add that when CAF are abundantly present in the connective tissue stroma with high expression of FAP-α they present with more invasive capability with an increase in Tumor budding activity and Lympho-Vascular invasion which might result in Lymph-node metastasis.

CONCLUSION

Our study tried to co-relate expression of FAP α in OSCC with lymph node metastasis.

In normal physiologic states, the expression of FAP α is absent whereas during induction and proliferation of tumor cells, FAP α expression is seen both in the epithelial as well as the connective tissue component.

The expression of FAP-α is seen in CAF present in the connective tissue stroma and tumor cells proliferating into and within the connective tissue stroma. This is a first kind of study done in OSCC co-relating the expression of FAP α with lymph node metastasis and other histopathological parameters. We found there is significant association noted between percentage of FAP α positive CAF and status of lymph node metastasis. Significant association was also noted between percentage of FAP α +ve CAF and increase in tumor budding, lympho-vascular invasion status in our study sample.

Collaborating the findings in this study we will like to conclude, FAP- α has definite role in progression of OSCC and its expression can be a diagnostic clue in evaluating the progression rate of OSCC. FAP α +ve CAF can also be a therapeutical target in clinical treatment of OSCC.

FUTURE SCOPE

Cancer-associated fibroblasts are been increasingly viewed as a target that could be manipulated and targeted for therapeutic benefit in patients with various cancer. There are now many clinical trials involving CAF-targeting agents in

combination with existing therapies. The underlying rationale for which is that by targeting CAF, there will be improvements in the access of either conventional therapies or T cell response towards the tumour.

LIMITATIONS

1. The limited sample size in the subgroups may have affected some of the findings.
2. Follow up data for all the participants was not available

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ANNEXURE I

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
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FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1462

CERTIFICATE

This is to Certify that the synopsis titled

Expression of fibroblast activation protein-alpha in oral squamous cell carcinoma and its association with lymph node metastasis - An immunohistochemical study *Submitted by*

Dr. [redacted] *P. G. Student /*

Staff, Guided by [redacted] *from Department of*

Oral and Maxillofacial Pathology & Oral Microbiology *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE II

BIOSTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of [ ] **Post Graduate Student**, under the guidance of **Dr.** [ ] **Reader, Department of Oral Pathology and Microbiology**, entitled **"EXPRESSION OF FIBROBLAST ACTIVATION PROTEIN-ALPHA IN ORAL SQUAMOUS CELL CARCINOMA & ITS ASSOCIATION WITH LYMPH NODE METASTASIS- AN IMMUNO-HISTOCHEMICAL STUDY"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 15.12.2022

Name & Signature of Biostatistician
Dr. S.B. Javali
Sr. Asso. prof. in statistics
Dept. of com. medicine
USM KLE IMP, Belagavi.

ANNEXURE III

WAIVER FORM

Department Of Oral and Maxillofacial Pathology and Oral Microbiology

KAHER VK Institute of Dental Sciences, Nehru Nagar, Belagavi-10

"Expression of Fibroblast Activation Protein-α in Oral Squamous Cell carcinoma & its Association with Lymph Node Metastasis"

Waiver of informed consent form

It is not feasible to obtain individual informed consent of donors of specimens used in this study. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/publication arising from the study.

ANNEXURE IV

PREPARATION OF APES (3- AMINO PROPYL TRIETOXYSALINE)

COATED GLASS SLIDES:

1. Clean dried glass were dipped in 1 % APES in acetone.
2. Slides were drained and dipped in acetone
3. Slide were drained again and dipped in dipped in distilled water.
4. Slides were then placed in a rack and allowed to dry.
5. Slides can then be stored and used as required.

ANNEXURE V

HEMATOXYLIN AND EOSIN STAINING TECHNIQUE (REGRESSIVE)

1. Sections were de-parrafinized by warming on slide warmer for 10 min and passed through Xylene I and Xylene II for 10 min each.
2. Slides were passes through 90 % and 70% alcohol for 5 min each.
3. Slides were rehydrated by keeping in running water for 10 min.
4. Slides were dipped in Harris hematoxylin for 3 minutes 30 seconds.
5. Slides were kept in water wash for 2-3 min.
6. Slides were differentiated by 1 dip in 1% acid alcohol
7. Slides were kept in water wash for 10 min.
8. Bluing was done by keeping the slides in lithium carbonate for 5-8 min and then water wash for 10 min
9. Slides were stained with eosin 10 sec.
10. Slides were passed through increasing grades of alcohol, 70% and 90% for 5 sec each.
11. Slides were dried, cleared in xylene and mounted.

ANNEXURE VI

PHOSPHATE BUFFER SALINE: It is used as wash buffer with pH ranging from 7.2-7.6.

The preparation formula is as follows:

1. Sodium Chloride (NaCl) – 3.2gm
2. Di-potassium hydrogen phosphate - 0.484 gm
3. Potassium di-hydrogen phosphate-0.144 gm
4. Dissolve the salts to make the volume up to 500 ml by adding distilled water.
5. The solution can be stored in a clean amber colored bottle in the refrigerator for a week.