QUANTIFICATION OF LARGE TUMOR SUPPRESSOR KINASE 1 AND 2 IN TOBACCO ASSOCIATED POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA

Dissertation submitted to

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

In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

2017-2020

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

CHENNAI

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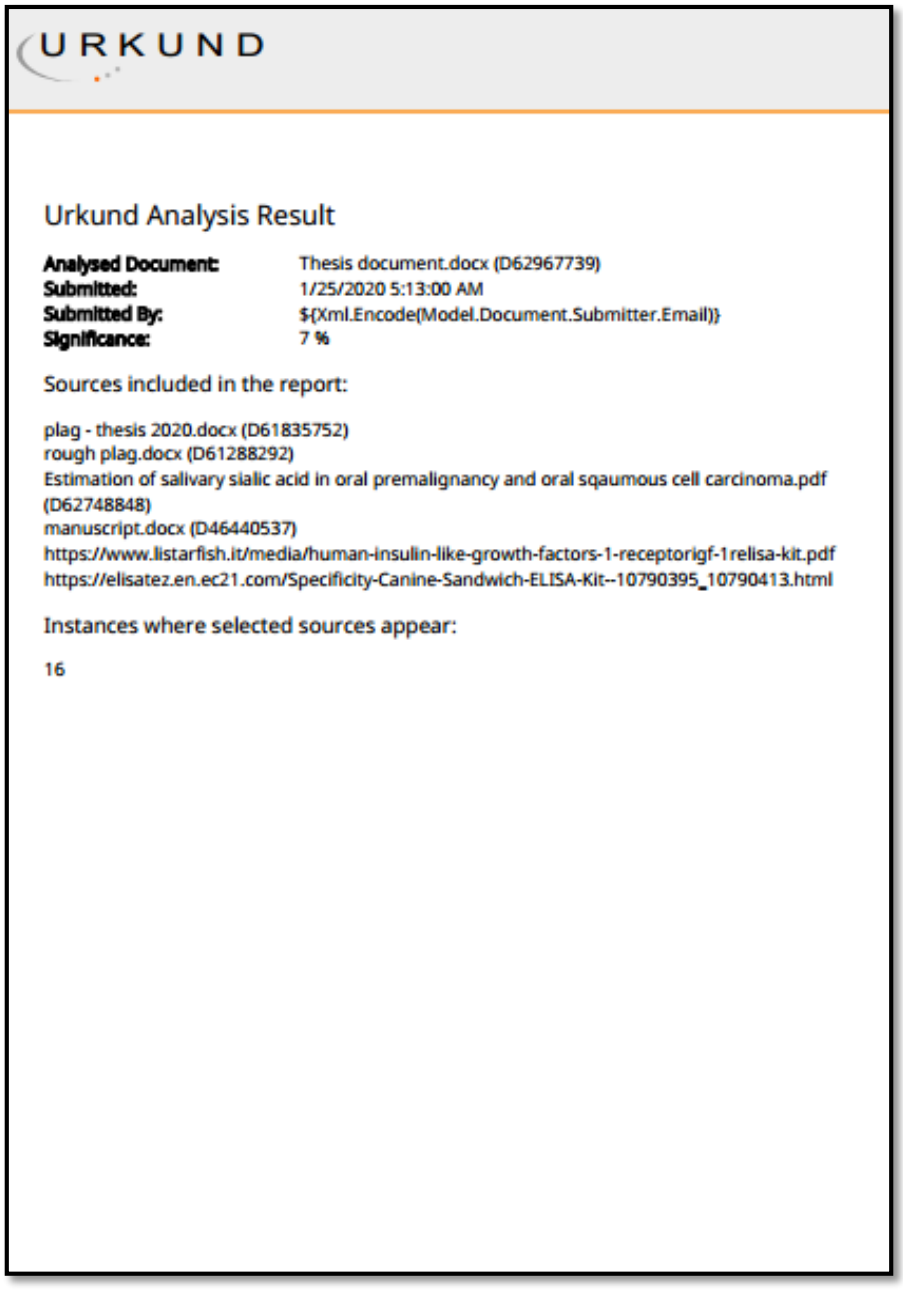

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ACKNOWLEDGEMENT

I sincerely thank **Dr. P. RAJESH, M.D.S., DNB, MNAMS, MFDSRCPS,** Principal, Chettinad Dental College & Research Institute, for the guidance, timely encouragement and the infrastructure provided for pursuing my course.

I extend my sincere gratitude to **Dr. R. Sathish Muthu Kumar, M.D.S,** Head of the department, Department of Oral and maxillofacial pathology for the guidance, encouragement, support, extreme patience, tireless help and time spent in correcting and completing the book. Thank you sir.

I sincerely thank **Dr. Sreeja. C, M.D.S,** Department of Oral and maxillofacial pathology, for the guidance, timely moral support, overwhelming help, advice and encouragement throughout the thesis correction.

I sincerely thank **Dr. R. Murugesan, P.H.D.,** Research director, Faculty of Allied health Sciences (FAHS), Chettinad Academy of Research and Education (CARE), for providing the adequate lab facilities and guidance in initiating the thesis work.

I sincerely thank **Dr. P. Surajit Pathak, M.Sc., P.H.D.,** Associate professor, Department of genetics, Faculty of Allied health sciences (FAHS), Chettinad Academy of Research and Education (CARE), for being kind, guiding and teaching the lab procedures and timely help in completing the thesis procedure.

I extend my sincere thanks to **Dr. Antara Banerjee,** Assistant professor, Department of genetics, Faculty of Allied health sciences (FAHS) Research director, Chettinad Academy of Research and Education (CARE), for the cheerfulness and kindness.

I sincerely thank **Dr. Nachiammai, M.D.S,** and **Dr.Merlin Jayaraj, M.D.S,** Department of Oral and maxillofacial pathology, for their extreme

support, kindness, suggestions on improving my thesis and the time spent in correcting my entire book in the busy schedule.

I sincerely thank **Dr.Harini Priya.S, M.D.S,** and **Dr.Serena williams, M.D.S,** Department of Oral and maxillofacial pathology, for their encouragements, corrections and continuous supervision in completing thesis work.

I sincerely thank **Dr. Govindarajan,** Statistician, for the data analysis, patience and comfortable space provided for thesis completion.

I Sincerely thank **Dr. Jaganath, Dr. Ganesh, Dr. Sarobala and Dr. Meenu**, Research scholars, Allied health sciences, Chettinad Dental College & Research Institute for their enormous help and guidance throughout the lab procedures.

I would also like to sincerely thank my well wisher **Mrs. Vijaya,** lab technician, Department of Oral and maxillofacial pathology for her support and also all the lab technicians, Department of microbiology, for their help and support.

My sincere gratitude to **Mr. Thiagu** and **Mrs. Kavitha,** Netway prints, Porur for their response and dedication in aligning my entire thesis book.

I sincerely thank my colleague **Dr. A. Beeula Raja kumari** and juniors **Dr. Anu Priya**, **Dr. Krithigaa, Dr. Leo Caroline** and **Dr. Precilla Pavelo** for their love, continous support and help in all my endeavors.

I sincerely thank my parents **Mr. K. Santha kumar** and **Mrs. S. Revathi** and my brother **Mr. Aswin Kumar. S** for their encouragement and enormous support in all my endeavors.

I thank the almighty for everything I have.

ABSTRACT

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the 11th most common cancer in the world. Consumption of tobacco and its products contribute for more than 90% of the OSCC. Early detection of OSCC at its precancerous stage greatly increases the chances for successful treatment. Tumor markers are proteins that are present and/or produced in high amount by malignant cells which provides information on the biological behaviour of cancer- its aggressiveness, response to therapy and targeted treatment. Literature confirms that, large tumor suppressor kinase 1 and 2 (LATS1 & LATS2) are the upstream tumor suppressor kinases in hippo signalling pathway that plays a significant role in oral squamous cell carcinoma. This study was attempted to elucidate the expression pattern and the role of large tumor suppressor kinase 1 and 2 in the disease process of potentially malignant disorder and oral squamous cell carcinoma in saliva using ELISA test.

AIM

To quantify large tumor suppressor kinase 1 and 2 in tobacco associated potentially malignant disorder and oral squamous cell carcinoma.

MATERIALS AND METHODS

This study consists of a total of 40 subjects comprising 10 normal controls (Group I), 10 subjects of smokers without lesion (Group II), 10 subjects with tobacco associated potentially malignant disorder (Group III) and 10 subjects with tobacco associated oral squamous cell carcinoma (Group IV). Saliva samples was collected from all 40 subjects and the expression of large tumor suppressor kinase 1 and 2 were quantified using ELISA test.

STATISTICAL ANALYSIS

One way ANOVA test was used to analyse the expression of LATS1 and LATS2 in study groups and also among various cytological grades. Independent sample t-test was used to analyse the expression of LATS1 and LATS2 among the histopathology grades of oral squamous cell carcinoma subjects (Group IV).

RESULTS

Group I (Normal control) had a mean LATS1 and LATS2 value of 2.7047ng/ml and 9.9093ng/ml respectively, Group II (Smokers without lesion) had a mean LATS1 and LATS2 value of 3.2828 ng/ml and 7.6075ng/ml respectively, Group III (Potentially malignant disorder) had a mean LATS1 and LATS2 value of 4.5442ng/ml and 10.8238ng/ml respectively and in Group IV (Oral squamous cell carcinoma) the mean LATS1 and LATS2 value was 5.0524ng/ml and 3.0849ng/ml respectively.

CONCLUSION

LATS1 and LATS2 proteins are sufficiently expressed in saliva. Its expression in saliva can be valuable in observing the disease transformation from a potential malignant disorder to carcinoma. This may help in the early prediction of oral squamous cell carcinoma at its precancerous stage and also in monitoring the disease process.

Key words: LATS1, LATS2, Tumor suppressor, smokers with no lesion, potentially malignant disorder and oral squamous cell carcinoma.

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ABBREVIATION	EXPANSION
4-ABP	4-aminobiphenyl
ADAM9	ADAM A Disintegrin and Metalloprotease
ADAMTS13	ADAMTS A Disintegrin and Metalloproteinase with Thrombospondin motifs
AJCC	American joint committee on cancer
ANKRD1	Ankyrin repeat domain-containing protein 1
ANOVA	Analysis of variance
anti-OVA IgG	Anti-Ovalbumin specific IgG
AP-1	Activator Protein 1
BaP	Benzopyrene
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CCK-8 assay	Cell Counting Kit -8
CLL	Chronic lymphocytic leukemia
CRB1	Crumbs Cell Polarity Complex Component 1
ccRCC	Clear cell renal cell carcinoma
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich angiogenic inducer 61
DAPK1	Death associated protein kinase 1
DMBA	7,12-dimethylbenzanthracene
DNA	Deoxyribonucleic Acid

ELISA	Enzyme linked Immunosorbent Assay
ERBB4	erb-b2 Receptor tyrosine kinase 4
FRMD6	FERM Domain containing 6
GCF	Gingival Crevicular Fluid
GLOBOCAN	Global Cancer institute
HNSCC	Head and neck squamous cell carcinoma
HPV	Human Papilloma Virus
HRP	Horse radish peroxidase
IARC	International Agency for Research on Cancer
ICD	Intracellular Domain
IHC	Immunohistochemistry
LATS1 & 2	Large Tumor Supressor kinase 1 and 2
LPA	Lysophosphatidic Acid
Maspin	Mammary Serine Protease Inhibitor
MC38	Murine colon cancer cell line
MMP	Matrix metalloproteinase
MNBA	4-(Methylnitrosamino) Butyric Acids
MNPA	3-(Methylnitrosamino) Propionic Acids
MOB1A	MOB kinase activator 1A
MOB1B	MOB kinase activator 1B
NDMA	Nitrosodimethylamine
NF2	Neurofibromin 2
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N′-nitrosonornicotine

NSAR	N-nitrososarcosine
NSCLC	Non small cell lung carcinoma
OPMD	Oral Potentially Malignant Disorders
OSCC	Oral Squamous Cell Carcinoma
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PPOELs	Potentially premalignant oral epithelial lesions
RASSF1	Ras Associated domain family member 1
RhoA/ROCK pathways.	Rho-Associated protein kinase
RNA	RiboxynucleicAcid
RT-PCR.	Real time PCR
RUNT1,2	Runt domain of RUNX1
RUNX	Runt-related transcription factor
SAS	Oral squamous cell carcinoma cell line
SAV	Salvador
SCC 25	Oral Squamous Cell Carcinoma Cell line
SD	Standard deviation
SMAD	Mothers against decapentaplegic
SMG	Submandibular Salivary Gland
SNAIL	SNAI 1, a zinger-finger transcriptional protein
STK3	Mammalian STE20 -like kinase2(MST2)
STK4	Mammalian STE20 -like kinase1(MST1)
Streptavidin HRP	Streptavidin Horse Radish Peroxidase

TAZ	Transcriptional co activator with PDZ-binding motif
TBX5	T-Box Transcription Factor 5
TEAD	TEA Domain family member
TGF-β	Transforming Growth Factor-β
TSNA	Tobacco-Specific N-nitrosamines
WHO	World Health Organization
WWC1	WW domain-Containing protein 1
WWTR1	WW domain containing Transcription Regulator
YAP1	Yes Associated Protein-1

INTRODUCTION

Oral squamous cell carcinoma is a multifactorial disease, many of which are attributed to tobacco and alcohol usage.[1]Consumption of tobacco and its products contribute for more than 90% of the OSCC[2] and is considered as an important risk factor for premature death globally. India is one among the few countries where both tobacco smoking and smokeless tobacco usage is high.[3]

About more than 8,000 chemical compounds have been identified in smoke and smokeless tobacco forms. Among these, about 70 chemicals have been found to be carcinogenic by International Agency for Research on Cancer (IARC). In cigarettes there are about six important carcinogens; benzo[*a*]pyrene (BaP), 4 (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N′*-nitrosonornicotine (NNN), *N*-nitrosodimethylamine (NDMA), ethylene oxide, and 4-aminobiphenyl (4-ABP).[4] The two important carcinogens present in smokeless tobacco are non-volatile alkaloid-derived tobacco-specific N-nitrosamines (TSNA) and N-nitrosoamino acids.[5]

Tobacco in human body induces sustained inflammation, compromised immune status, diabetes mellitus, respiratory related symptoms, cardiovascular diseases and oral squamous cell carcinoma.[6] Epidemiological research suggests that epithelial lesions are the most common source of malignancy.[7] Tobacco usage mainly affects the surface epithelium, resulting in changes in the appearance of the tissues. The changes may range from an increase in pigmentation

to a significant thickening of the epithelium or a distinct white lesion.[8]

Tobacco causes few specific alterations in the individual squamous epithelial cell resulting in 'epithelial dysplasia'.[9]Epithelial dysplasia is described as an anomaly of growth, produced by abnormal or atypical epithelial proliferation resulting in a lesion with disturbed differentiation and maturation.[10]Epithelial dysplastic changes are the hallmark of oral potentially malignant disorders (OPMDs) observed during histopathological examination.[7]Dysplasia precedes oral squamous cell carcinoma[9] of which, about less than 5% are mild dysplasia, 30% are moderate dysplasia and 50% are severe dysplasia. Literatures state that the different grades of dysplasia progress successively to oral squamous cell carcinoma, although not proven.[11]

The world wide prevalence rate of OPMDs are 1-5%.[12] OPMDs induced by tobacco include leukoplakia, erythroplakia, palatal lesions in reverse smokers, oral submucous fibrosis, actinic keratosis, lichen planus and discoid lupus erythematosus.[13]Leukoplakia is the most common,[14]whose prevalence in India varies from 0.2% to 5.2% with a malignant potential of 0.13% to 10%.[15]Oral leukoplakia and erythroplakia are associated with both smoke and smokeless forms of tobacco. Among the smokeless tobacco consumers, buccal mucosa and mandibular buccal vestibule are commonly affected sites due to the placement of areca nut and tobacco quid.[16, 17]Apart from the addictive habit many other factors like genetic factor, existence of dysplasia and

candidal infections are involved in the malignant transformation of leukoplakia.[18]

Oral squamous cell carcinoma (OSCC) is the 11th most common cancer in the world.[12] According to GLOBOCAN 2018 (Global cancer institute), it is the 2nd most common cancer in India.[19]About 5 people in India die every hour each day due to OSCC. The International Agency of Research on Cancer (IARC) has estimated the rise in incidence of OSCC from 1 million in 2012 to 1.7 million in 2035. The incidence of oral cancer among male is 28% and female is 52% whereas the mortality rate is 6.8% and 3.6% per 100,000 population respectively.[20]

The cell division, its differentiation and death of the oral squamous epithelium are regulated by various excitatory and inhibitory pathways which are in turn governed by the genes. These are two classes of genes known as the proto-oncogenes and tumor-suppressor genes encoding various kinds of proteins that controls the cell growth and proliferation. Mutations of these genes contribute to the development of cancer [21] altering the regulatory pathways.

Hippo signalling pathway is one of the important growth regulatory pathway. It modulates cell proliferation [22]and plays an important role in regulating organ size and growth by co-ordinately controlling the cell proliferation and survival.[23] Large tumor suppressor kinase (LATS1 & LATS2) are the central regulators of cell fate, genome integrity and prevents cancer by modulating the functions

of numerous oncogenic or tumor suppressive effectors in hippo signalling pathway.[24]

Hence this study was designed to quantify the salivary levels of Large tumor suppressor kinase 1 and 2 in normal controls, smokers, tobacco associated potentially malignant disorder and oral squamous cell carcinoma by ELISA test. This might help in understanding the role played by LATS1/2 in oncogenic process and thereby aiding in early diagnosis and management of oral squamous cell carcinoma. Furthermore, most studies employ only one *LATS* gene or protein, making it difficult to identify true differences between LATS1 and LATS2. In this study, we have tried to analyse the discrete characteristics of LATS1 and LATS2, in the progression of oral squamous cell carcinoma (OSCC).

AIM AND OBJECTIVES

AIM

To quantify large tumor suppressor kinase 1 and 2 in tobacco associated potentially malignant disorder and oral squamous cell carcinoma.

OBJECTIVE

- To quantify the large tumor suppressor kinase 1 and 2 (LATS1& 2) in saliva of 4 different groups of patients.
- To correlate the levels of large tumor suppressor kinase 1 and 2 with the clinical parameters of potentially malignant disorder and oral squamous cell carcinoma.
- To enlighten the role of large tumor suppressor kinase 1 and 2 in initiation and progression of oral squamous cell carcinoma.

3.1 LARGE TUMOR SUPPRESSOR KINASE 1 AND 2

INTRODUCTION

Hippo signalling pathway, a novel signalling pathway was first found in a fruit fly called "Drosophila melanogaster". It has an important role in controlling cell proliferation and promotes apoptosis in differentiating epithelial cells. The pathway was studied in interest to know the control of cell numbers during normal organ development. At present, it is considered as a liable target for misregulation in cancer.[24]

Genetic studies in Drosophila melanogaster reveal many genes with mammalian homologues that function as tumor suppressor genes and oncogenes which are part of the hippo signalling pathway.[24] In humans, deregulation in the pathway leads to increased cellular proliferation, inhibition of apoptosis and deregulation of cellular differentiation which are the key hallmarks in cancer development.[25]

HIPPO COMPONENTS

Hippo signalling pathway genes were initially identified in the fruit fly Drosophila melanogaster using genetic mosaic models, which are Hpo, Wts, Mats, Mer, Yki, Sd, Kribra, Crb, and Ex. The orthologs to these components were subsequently found in mammals, they are STK4, STK3, LATS1, LATS2, MOB1A, MOB1B, NF2, SAV, YAP1,WWTR1,TEAD, WWC1,CRB1, FRMD6, and RASSF1.[26,27] Among these, the most common genes involved in oral carcinogenesis are listed in (Table 1).

Table 1: Core components of Hippo signalling pathway [5]

DORSOPHILA MELANOGASTER		HUMAN ORTHOLOGS		FUNCTION
GENE	**PROTEIN**	**GENE**	**PROTEIN**	
Hpo(2003)	Hippo	STK4	Mammalian STE20 -like kinase1 (MST1)	Clarified on novel kinase cascade and transcriptional genes like Diap 1 and Cyclin E
		STK3	Mammalian STE20 -like kinase2 (MST2)	
Wts(1995)	Warts	LATS1 (1999)	Large tumor suppressor kinase1	➢ Loss of Wts function results in tissue overgrowth ➢ LATS1deficiency cause development of soft tissue sarcoma and ovarian tumors in Lats1 deficient mice ➢ Identification of LATS phosphorylation sites(2007)[29]
		LATS2	Large tumor suppressor kinase2	
Mats(2005)	Mats	MOB1A	MOB kinase activator 1A	Binds to Warts and potentiates kinase activity
		MOB1B	MOB kinase activator 1B	
Mer(2006)	Merlin	NF2	Neurofibromin-2/Merlin	An upstream component of hippo signalling pathway
Sav(2001)	Salvador	SAV1	Salvador homolog 1	First binding partner of Sav
Yki(2005)	Yorkie	YAP1	Yes associated protein 1/YAP	Nuclear effector of hippo pathway
		WWTR1	WW domain containing transcription regulator 1/TAZ	
Sd(2008)	Scalloped	TEAD 1	TEA domain family member 1	DNA binding partner and mediator in organ size control
		TEAD2	TEA domain family member 2	
		TEAD3	TEA domain family member 3	
		TEAD4	TEA domain family member 4	

REGULATION OF HIPPO SIGNALLING PATHWAY

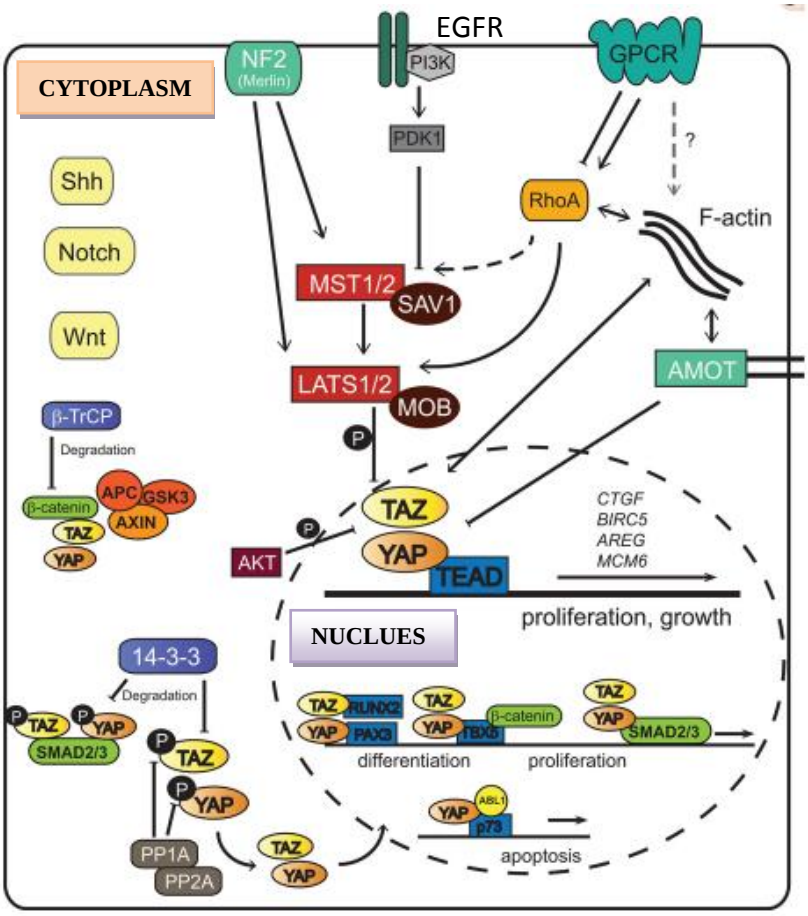


Fig 1: Hippo signalling pathway in mammals[30]

The mammalian Hippo pathway consists of a protein complex that includes MST1 and MST2, large tumor suppressors 1 and 2, adaptor proteins SAV1 and MOB kinase activator 1A and 1B kinases.[31]MST1 and MST2 are activated by Hippo,[21] which in turn facilitate LATS1 and LATS2 phosphorylation, followed by LATS-dependent phosphorylation of the homologous oncoproteins YAP and TAZ.

In an active Hippo pathway, its main effectors, YAP and TAZ, are phosphorylated by the Hippo core complex and retained in the cytoplasm where they undergo proteosomal degradation. This

facilitates tumor suppressor activity when located in the cytoplasm. Inactive or suppressed Hippo pathway leads to YAP and TAZ translocation to the nucleus. In the nucleus, they function as oncogenes by facilitating transcriptional activation and regulates the activity of various transcription factors such as SMADs, TEADs, TBX5, and RUNT1,2 and p73 are involved in cell proliferation, survival, microRNA processing, metastases and stem cell maintenance. Based on this mechanism, Hippo effectors YAP and TAZ can act either as a tumor suppressor or as an oncogene.[31]

YAP and TAZ can bind to transcription factors other than TEADs, such as AP-1 (activator protein 1) to activate genes involved in S-phase entry in epithelial cells, the intracellular domain (ICD) of ERBB4 (erb-b2 receptor tyrosine kinase 4), SMADs links to transforming growth factor-β (TGF-β) pathways, p73 causes transcription of p73 proapoptotic target genes- p53AIP1 and BAX and also with transcription factors of the RUNX family.[32] (Fig 1)

MUTATION

Normal oral epithelial cells are tightly controlled and regulated by various excitatory and inhibitory pathways which contributes to the basic cellular functions like cell division, differentiation, senescence and adhesion.[33]One among the most important regulatory pathway is hippo signalling pathway. Various carcinogens like tobacco, alcohol and dietary carcinogens alters the normal binding of extra cellular ligands of the regulatory pathways to the cell-surface receptors which

leads to subsequent alterations in the intracellular signals that are sent through secondary messengers to the cell.[33]

These chromosomal alteration or mutations in genetic materials (proto-oncogenes/oncogenes and tumor suppressor genes) accumulates within the cell and are converted into tumor cells leading to proliferation, neo-vascularisation and progression of oral cancer.[33, 20]

TOBACCO

Tobacco consumption is a very serious potential health hazard and a reason for morbidity and mortality worldwide. Numerous tobacco products are available in various consumption patterns. Tobacco can be broadly classified as smoking and smokeless tobacco where the later can be consumed orally and nasally.[34]

TOBACCO AND ITS CARCINOGENS

The main component of tobacco is nicotine which is not carcinogenic but is responsible for tobacco addiction.[35]

Smokeless tobacco

The common and the major group of carcinogens are the non-volatile alkaloid-derived tobacco-specific N-nitrosamines (TSNA) and N-nitrosoamino acids. The non-volatile TSNA, includes 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) and N-nitrosoamino acids includes N-nitrososarcosine (NSAR), 3-(methylnitrosamino) propionic acids (MNPA) and 4-(methylnitrosamino) butyric acids (MNBA). In India, N.

tabacum species contains up to 0.035µg/g NNN and 0.0115µg/g NNK and N. rustica species contains up to 46.1 µg/g NNN and 2.34 µg/g NNK in.[5]

Other carcinogens are volatile N-nitrosamines, certain volatile aldehydes, traces of few polynuclear aromatic hydrocarbons, urethanes, benzopyrene, certain lactones, urethane, metals, polonium-210 and uranium-235 and 238.[5]

Tobacco smoke

It contains polycyclic aromatic hydrocarbons (PAHs) such as Benzopyrene (BaP) and 7,12-dimethylbenzanthracene (DMBA) alike car exhaust and furnace gases.[36]

ROLE OF TOBACCO IN ORAL CANCER

Schmidt BL, Dierks EJ, Homer L et al (2004),[37] in a retrospective study examined the relationship of smoking versus never smoking history in oral squamous cell carcinoma patients. They correlated it with stage and site presentation among 67 patients of oral squamous cell carcinoma. It was found that 33% patients were never smokers and 67% of patients were smokers with an average of 49.4 pack-years. The study concluded a strong association between smoking history and carcinoma involving the posterolateral tongue and floor of mouth.

Karine A Al Feghali, Ahmed I Ghanem, Charlotte Burmeister et al (2019),[38] in a retrospective study to determined the relationship between non-smoking (N=55) and smoking (N=108) associated oral

squamous cell carcinoma (OSCC) to adverse pathological features in 163 patients with AJCC stage I–IVa cancer (2005-2015). The study inferred that smokers were more likely to have advanced disease stage and tumors with aggressive pathological features than non-smokers. Three-year actuarial overall survival was 62% and recurrence free survival was 68% in smokers and 81% and 69% in non-smokers respectively.

TOBACCO AND ITS ASSOCIATION WITH HIPPO SIGNALLING PATHWAY

Yue Zhao, Wei Zhao, Liyan Xue et al (2014),[39] studied in T3 stage oesophageal squamous cell carcinoma tissue specimens of 83 patients consisting of 29 non-smokers and 54 smokers. Immunohistochemical analysis revealed strong upregulation of YAP1 (60%) in 49 cancer samples. The inference also showed a significant association between YAP1 upregulation and cigarette smoking concluding the oncogenic activation of YAP1.

ROLE OF LATS1/2 IN OSCC

Expression of LATS1 inhibits oral squamous cell carcinoma

Reddy et al (2015),[40]in a study investigated LATS1 DNA in 13 biopsy tissues of oral squamous cell carcinoma by PCR. They found that 54% of oral squamous cell carcinoma tissues had LATS1 promoter hypermethylation. The study concluded that hypermethylation of LATS1 can be one of the early event in carcinogenesis.

Expression of LATS2 inhibits oral squamous cell carcinoma

Yue ZW et al (2017),[41] studied the role of mRNA expression and promoter methylation of LATS2 in 72 OSCC specimens and normal oral mucosa using RT-PCR. The study revealed down regulation of LATS2 mRNA in normal oral mucosa, 47% of OSCC showed the expression of LATS2 mRNA and about 68% of OSCC showed promoter methylation. The study concluded that promoter methylation of LATS2 gene may play a significant role in the occurrence of OSCC.

Yue ZW Wang et al (2018),[42] investigated the effect of large tumor suppressor kinase 2 in proliferation and apoptosis of oral squamous cell carcinoma using OSCC cell line (SCC 25) in western blot. The cell proliferation was assessed using CCK-8 assay and the apoptosis was detected using flow cytometry. The study concluded that overexpression of LATS2 inhibits the proliferation and apoptosis of the SCC 25.

Expression of LATS 1/2 progresses oral squamous cell carcinoma

Mohammad Ayoub Rigi Ladiz et al (2017),[43] analysed the promotor methylation and mRNA levels of LATS1/2 genes in 70 paraffin embedded tissues and normal oral samples. They have found decreased levels of mRNA expression in OSCC and a significant association between methylation of LATS1/2 and risk of OSCC development.

Nozaki M et al (2019),[44] studied the expression of LATS1/2 in OSCC cell line (SAS). LATS1/2 were highly expressed in SAS cell line which induced higher proliferative capacity in these cancer stem cells.

LATS1/2 were more highly expressed before the initiation of self renewal and prevented SAS cells from forming spheres. Hence the study concluded that LATS1/2 triggers the self renewal of cancer stem cells.

Table 2: Role of Large tumor suppressor kinase in OSCC - A divergent view

AUTHOR	PROTEIN	SAMPLE SIZE	SPECIMEN TYPE	METHOD	INFERENCE
Reddy, et al (2015) [40]	LATS1 DNA	13	Biopsy tissues of OSCC	PCR	➢ 54% of OSCC tissues had LATS1 promoter hypermethylation ➢ Hypermethylation of LATS1 is one of the early event in carcinogenesis
Yue ZW, et al (2017) [41]	LATS2 (gene)	72	➢ OSCC specimens ➢ Normal oral mucosa	➢ RT-PCR ➢ Western blot	➢ LATS2 mRNA was down regulated in normal oral mucosa ➢ 47 % of OSCC expressed LATS 2 mRNA ➢ 68 % of OSCC has promoter methylation ➢ Promoter methylation of LATS2 gene play a significant role in the occurence of OSCC
Mohammad Ayoub Rigi Ladiz et al (2017) [43]	LATS1/2 (gene)	70	➢ OSCC tissues ➢ Normal oral sample	➢ Methylation specific PCR	➢ LATS1/2 was down regulated through promoter methylation in OSCC ➢ Significant association between methylation of LATS1/2 and the risk of OSCC
Yue ZW Wang, et al (2018)[42]	Large tumor suppressor kinase 2 (protein)	SCC-25 cell	OSCC cell line (SCC- 25)	➢ Western blot ➢ CCK-8 assay for assessing proliferation ➢ Flow cytometry to detect apoptosis	➢ Overexpression of LATS2 inhibits the proliferation and apoptosis of the SCC 25 cell
Nozaki M, et al (2019)[44]	LATS1/2(gene)	SAS cells	OSCC cell line (SAS cell)	➢ Low adhesion sphere culture dish	➢ Increased LATS1/2 expression in OSCC cell line SAS ➢ LATS1/2 highly expressed before the initiation of self renewal ➢ LATS1/2 triggers the self renewal of cancer stem cells

Table 3: Role of Large tumor suppressor kinase in other carcinomas- A divergent view

AUTHOR``	PROTEIN	SAMPLE SIZE	SPECIMEN TYPE	METHOD	INFERENCE
Godlewski J et al (2018)[45]	LATS1 (protein)	54	Renal tissues of clear cell renal cell carcinoma (ccRCC)	Immunohistochemistry	➢ LATS1 was observed only in the cytoplasm of tumor cells in 59.3% of patients. ➢ Decreased LATS1 immunoreactivity levels were associated with worse prognosis. ➢ LATS1 can be considered as new prognostic factors in ccRCC.
Esra SERT et al(2018)[46]	LATS2 (protein)	28 & 20	Blood samples of Chronic myeloid leukemia & controls	➢ RT-PCR ➢ Western blotting	➢ LATS2 gene expression is downregulated in CLL patient as compared to controls. ➢ LATS2 protein levels are down regulated in the CLL patient group
Celine Montavonet, et al (2019)[47]	LATS2 (protein & gene)	196	Ovarian tumor slides	➢ Immunohistochemistry ➢ Cell culture ➢ Western blot	➢ LATS is not an indicator of survival in serous ovarian cancer ➢ LATS was low in cystadenoma, intermediate in carcinoma, and high in borderline tumors and was higher in serous than mucinous ovarian carcinoma. ➢ LATS2 expression may even be tumorigenic.
Eojin Kim, et al (2019)[48]	LATS1/2 (protein)	318	Advanced gastric cancer tissues	➢ Tissue microarray ➢ Immunohistochemistry	➢ 223 (70.1%) cases were negative for LATS1/2 expression.

3.2 POTENTIALLY MALIGNANT DISORDERS

EVOLUTION OF TERMINOLOGY

In 1805- A panel of European physicians suggested that **oral precancer** are benign diseases which will always develop into invasive malignancies, if followed long enough.

In 1875- The term **'Precancer'** was first coined by Victor Babes, a Romanian physician.

In 1972- World Health Organizations (WHO) in 1972 subdivided 'precancer' into 'lesions' and 'conditions' with their definitions.[49]

In 1978- the WHO proposed the terms "**Precancerous conditions" and "Precancerous lesion"** and defined precancerous lesion as "a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart." Confusion prevailed over this terminology, and many opined that the prefix "pre" quotes that all precancerous lesions will eventually become cancer, whereas literature argues against this general belief.[50]

A much earlier working group of the **World Health Organisation** proposed in 1978 that clinical presentations of the oral cavity that are recognized as precancerous be classified into two broad groups, as lesions and conditions , with the following definitions:

Precancerous lesion is a morphologically altered tissue in which oral cancer is more likely to occur than in its apparently normal counterpart

Precancerous condition is a generalized state associated with a significantly increased risk of cancer.[51]

In 2005, IARC, WHO- The terms '**Pre-cancer'**, '**Precursor lesions'**, '**Premalignant'**, '**Intra epithelial neoplasia' and** '**Potentially malignant**' have been used in the international literature to broadly describe clinical presentations that may have a potential to become cancer. They all convey the concept of a two-step or multi-step process of cancer development, but it is unlikely, on a priori grounds, that there is uniformity in the way individual patients or tissues behave. 2005, WHO monograph, uses the term 'epithelial precursor lesions'.(51)

In 2005, the WHO - proposed to use the term **"Oral potentially malignant disorders"** (OPMDs), which is defined as "the risk of malignancy being present in a lesion or condition either at the time of initial diagnosis or at a future date."(50)

In 2007, the WHO- it was also recommended that the distinction between potentially malignant lesions and conditions can be abandoned in favour of a common term, **Oral potentially malignant disorders (OPMDs),** and this has now been accepted in the latest WHO classification.(52)

In 2007, the WHO, The consensus of the present working group was to recommend the term potentially malignant disorders, as it conveys that not all lesions and conditions described under this term may transform to cancer, rather that there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation.(51)

Paul M Speight 2018, proposed a new term **"Potentially premalignant oral epithelial lesions (PPOELs)"**. The underlying

concept is that these lesions have the potential to become malignant, so in their current state, that is, before malignant transformation, they are still (potentially) premalignant.[53]

LEUKOPLAKIA

Leukoplakia is the most common potentially malignant disorder, whose prevalence in India varies from 0.2% to 5.2% with a malignant potential of 0.13% to 10%.[14,15]

Definition

The various definitions for leukoplakia are,

In 1978, the WHO- A white patch or plaque that cannot be characterized clinically or pathologically as any other disease.

In 1984, in an international seminar- the definition was changed to "leukoplakia is a whitish patch or plaque that cannot be characterized clinically or pathologically as any other disease and it is not associated with any physical or chemical causative agent except the use of tobacco."[54]

In 1994, in an international symposium in Sweden-A predominantly white lesion of the oral mucosa that cannot be characterized as any other definable disease.[54]

In 2005, the WHO- defined leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer."[54]

Warnakulasuriya et al- Leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer.[51]

Clinical presentation

Depending on the clinical presentation, leukoplakia can be divided into homogeneous and non-homogeneous forms. The homogenous form displays a uniform pattern of reaction throughout the lesion, with a uniform white patch with shallow ridges in the epithelium. The non-homogeneous form is of three types: i) Speckled with mixed white and red appearance on the surface but predominantly white; ii) Nodular with small polypoid outgrowths which are rounded red or white outgrowths; and iii) Verrucous with a wrinkled or corrugated surface appearance.[55]

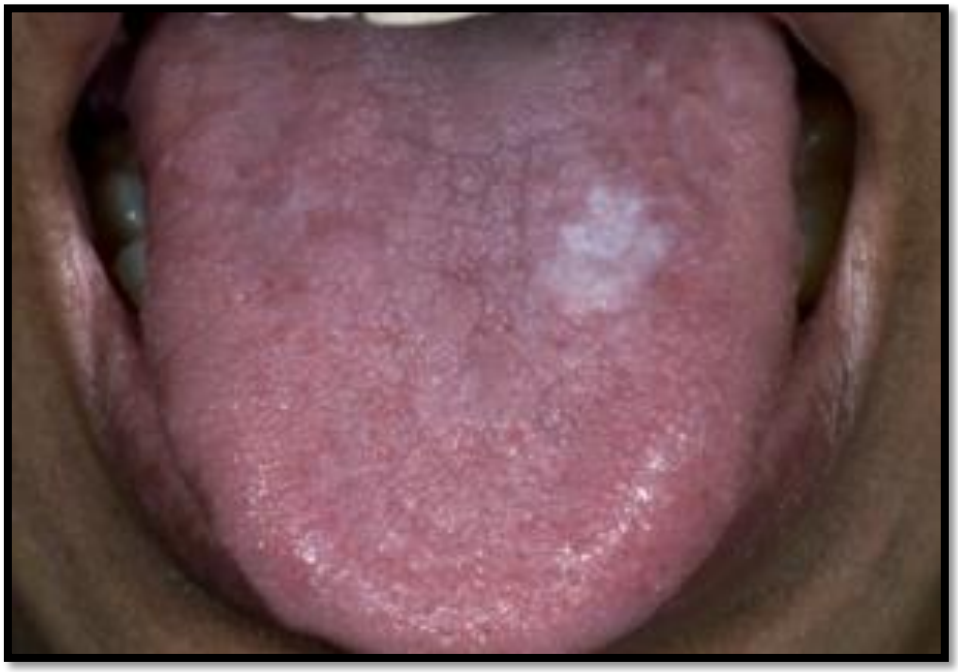


Fig 2: Leukoplakia in the floor of the mouth[56]

Table 4: Staging of oral leukoplakia[57]

STAGE	GRADING
L1: Single or multiple lesions together <2 cm. L2: Single or multiple lesions together 2-4 cm. L3: Single or multiple lesions together >4 cm. P0: No epithelial dysplasia. P1: Mild to moderate epithelial dysplasia. P2: Severe epithelial dysplasia.	Stage I: L1 P0. Stage II: L2 P0. Stage III: L3 P0 or L1/ L2 P1. Stage IV: L3 P1

Oral epithelial dysplasia

Dysplasia means abnormal growth. Dysplastic changes in the stratified squamous epithelium is characterized by cellular atypia, loss of maturation and stratification. There are several grading system for oral epithelial dysplasia. The first classification system was given in the year 1988.[58] (Fig 5 to Fig 8)

Table 5: Dysplastic features, WHO (2017)[58]

ARCHITECTURAL CHANGES	CELLULAR CHANGES
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (Nuclear pleomorphism)
Drop-shaped rete ridges	Abnormal variation in cell size (anisocytosis)
Increased number of mitotic figures	Abnormal variation in cell shape (Cellular pleomorphism)
Abnormal superficial mitosis	Increased nuclear-cytoplasmic ratio
Premature keratinization in single cells (dyskeratosis)	Atypical mitotic figures
Keratin pearls within rete ridges	Increased number and size of nucleoli
Loss of epithelial cohesion	Hyperchromasia

The cytological/cellular feature, “increase in nuclear size” and "Basal cell hyperplasia" in the 2005 WHO classification has also been removed in 2017 WHO diagnostic criteria of oral epithelial dysplasia. The architectural feature “loss of epithelial cell cohesion” has been included in 2017 WHO diagnostic criteria (Table 5).

Features of dysplasia

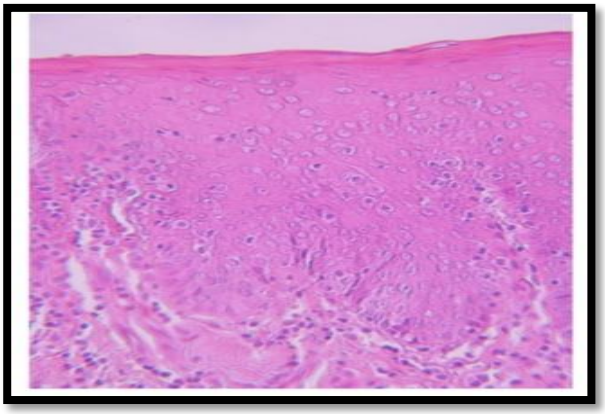


Fig 3: Irregular stratification

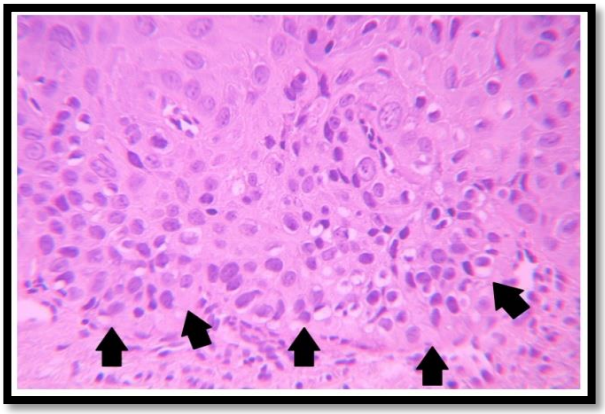


Fig 4: Loss of polarity of basal cells

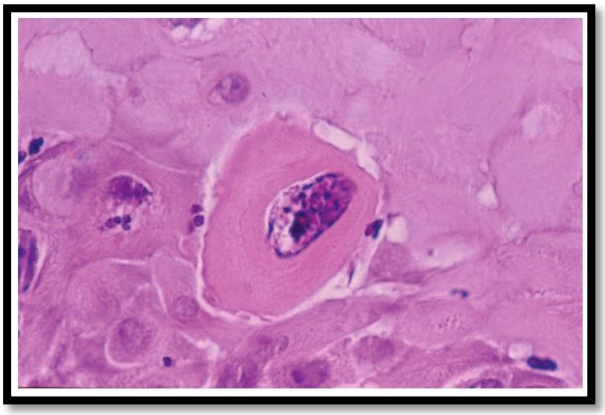


Fig 5: Dyskeratosis

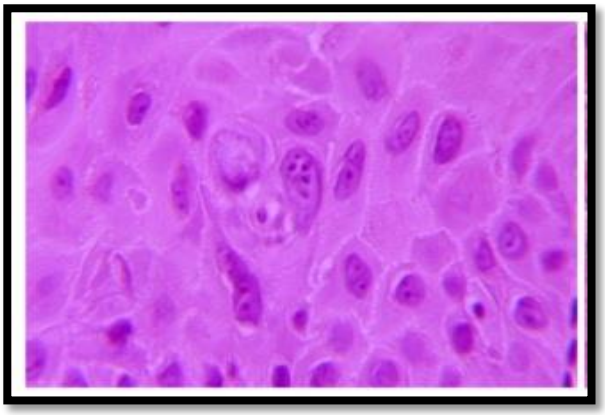


Fig 6: Anisonucleosis & Anisocytosis

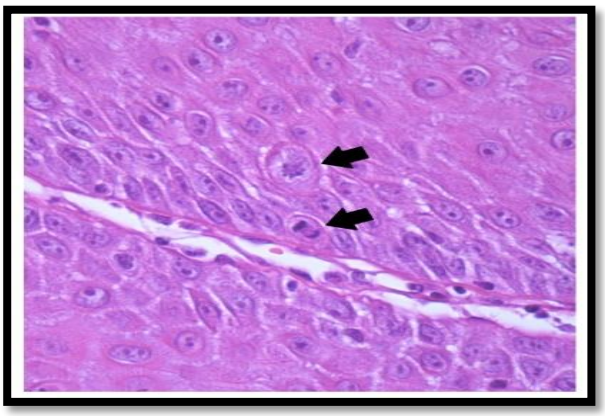


Fig 7: Mitotic figures

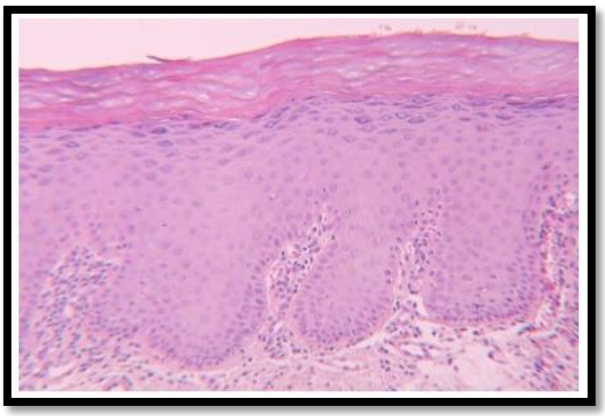


Fig 8: Hyperplasia & hyperkeratosis

3.3 ORAL SQUAMOUS CELL CARCINOMA

Oral cancer is traditionally defined as squamous cell carcinoma of the lip, oral cavity and oropharynx[59]because in the dental area, 90% of cancers are histologically originated in the squamous cells.[60]Oral squamous cell carcinoma (OSCC), is the most frequent with an estimated occurrence of more than 90% among oral neoplasms.[61]The major etiological and predisposing factor for oral squamous cell carcinoma are smoking, alcohol, ultraviolet radiation and other factors like human papillomavirus (HPV), Candida infections, nutritional deficiencies and genetic predisposition.[62]

Though oral cavity can be easily assessed for clinical examination, OSCC is usually diagnosed in advanced stages.[61] Lack of awareness among the public on the risk factors, signs, symptoms and preventive measures associated with oral cancer as well as absence of early detection by health-care providers are believed to be responsible for the diagnostic delay.[63]

EXAMINATION OF OSCC

According to World Health Organization (WHO), early detection of cancer greatly increases the chances for successful treatment. The two major approaches that could be followed by the health care providers for early detection of cancer is education on early diagnosis and screening.[64]

Screening of oral squamous cell carcinoma can be done by examining early signs and symptoms of cancer,[64]examining the changes at the cellular level (atypia) and at the resultant tissue level (dysplasia)[65]and also by molecular level to predict neoplastic potential of oral lesions and its malignant transformation.[66]Hence, keeping this in mind the Oral squamous cell carcinoma can be screened based on the following,

1. Clinical examination and staging
2. Cellular changes-Exfoliative cytology & Histopathology
3. Molecular tests- Proteins as tumor markers

1. Clinical examination

Oral precancer and cancer demonstrate a wide range of clinically detectable alterations that may range from an early subtle change in surface texture, color or elasticity to a more obvious lesion. Surface changes often have mixed red and white features with few, if any, associated symptom. Concomitant change in mucosal texture like firmness or induration on digital palpation, friability on slight manipulation and distortion of normal anatomy can be seen, while more advanced disease may feature fixation to surrounding and deeper tissues, often without pain or symptoms. Most early-stage lesions appear to be seemingly innocent alterations, in the form of focal color change (red, white or mixed), surface textural change (erosion, keratosis, granularity or fissuring) or both. These changes represent cellular alterations that result from genomic changes within the surface epithelial cell population. Such changes include alterations in DNA

content, loss of heterozygosity and genetic alterations in a stepwise progressive fashion that lead to formation of invasive squamous cell carcinoma.[67]

2. Exfoliative cytology

The superficial epithelial cells may exfoliate or abrade from the body surface as a result of physiological turnover mechanism. These exfoliated cells are quantitatively and qualitatively studied and inferred by the cytologist into following grades,[57]

Class I- Normal- Only normal cells are observed

Class II- Atypical- Presence of minor atypia but no evidence of malignant changes

Class III- Intermediate- Cellular atypia that may be suggestive of cancer, but not clear cut. They may represent precancerous lesions or carcinoma in situ. Biopsy is recommended.

Class IV- Suggestive of cancer- A few cells with malignant characteristics or many cells with borderline characteristics. Biopsy is mandatory.

Class V- Positive for cancer- Cells that are obviously malignant. Biopsy is mandatory.[57]

The advantages are it is simple, rapid, painless, non-invasive diagnostic technique[68]can be also used to follow up recurrent lesions.[69] The disadvantages are negative result are not reliable to exclude OSCC[70],inadequate cellularity of the smear[68], epithelial

tissue lesions only can be identified and tumor grading cannot be accessed [70]

3. Histopathological examination

Depending upon the degree of the resemblance to their parent tissue (squamous epithelium) and their normal product (keratin), the histopathological evaluation is done and is called as grading. The lesions are either graded on a 3-point or 4-point scale that is from grade I to III and from grade I to IV respectively. The histopathological grade of the tumor is related to the biological behaviour though the prognosis of the tumor is better correlated with the clinical staging than the histopathological grade.

Grade I or well differentiated OSCC- It is mature enough to closely resemble its tissue of origin and seems to grow at a slower pace and metastasize later in its course

Grade II or moderately differentiated OSCC- The microscopic appearance of tumor is somewhere between well differentiated and poorly differentiated OSCC.

Grade III or IV poorly differentiated OSCC- Cellular and nuclear pleomorphism and with little or no keratin production may be so immature that it becomes difficult to identify the tissue of origin. Such a tumor often enlarges rapidly, metastasizes early in its course.[71]

4. Tumor markers examination

A tumor marker have traditionally been proteins or can be anything that is present in or produced by cancer cells or other normal

cells but at a higher amount by cancer cells. Tumor markers are helpful in providing information about a cancer, such as how aggressive it is, whether it can be treated with a targeted therapy, or whether it is responding to treatment. Increasingly, however, genomic markers such as tumor gene mutations, patterns of tumor gene expression, and nongenetic changes in tumor DNA are being used as tumor markers.

Types of tumor markers

There are two main types of tumor markers that have different uses in cancer care: circulating tumor markers and tumor tissue markers.

(i) Circulating tumor markers can be found in the blood, urine, stool, or other bodily fluids of some patients with cancer. They are used to estimate prognosis, detect cancer that remains after treatment (residual disease) or that has returned after treatment, to assess the response to treatment and to monitor whether a cancer has become resistant to treatment.

(ii) Tumor tissue markers are found in the actual tumors themselves, typically in a sample of the tumor that is removed during a biopsy. They are used to diagnose, stage, and/or classify cancer, estimate prognosis and to select an appropriate treatment strategy.[72]

Ideal requirement of tumor marker

1. An ideal tumor marker should be highly sensitive, specific, reliable with high prognostic value, organ specificity and it

should correlate with tumor stages with 100% accuracy in differentiating between healthy individuals and tumor patients.

2. It should be detectable at early stage of tumor and should have prognostic value.
3. Its levels should be preceding the neoplastic process, so that it should be useful for screening early cancer. It should have high positive and negative predictive value.
4. It should be either a universal marker for all types of malignancies or specific to one type of malignancy and it should be easily assayable and be able to indicate all changes in cancer patients receiving treatment.[73]

3.4 SALIVA AND ITS APPLICATIONS

Saliva is a mucoserous exocrine secretion, secreted by the salivary glands. Salivary glands are broadly classified based on the anatomical size as major and minor salivary glands. The major salivary glands are parotid, submandibular and submental glands. The minor salivary glands are smaller salivary glands namely labial, lingual, palatal, buccal and glossopalatine glands

VOLUME: The average secretion of whole saliva ranges from 1-1.5 litres of which 20% is contributed by parotid gland, 65% by submandibular gland and 7-8% by the sublingual gland.[74]

COMPOSITION

Saliva is composed of electrolytes, immunoglobulins, proteins, enzymes, growth factors and buffers, attributing to various diagnostic applications (Table 6)

Electrolytes - Sodium, potassium, calcium, magnesium, bicarbonates and phosphates and hydrogen.

Immunoglobulins - IgA,IgG and IgM

Proteins-Gustin and Mucin

Enzymes- Alpha amylase, Lysozyme, lactoferrin, Sialoperoxidases, mucin glycoprotein's, agglutinins, histatins, proline rich proteins, statherins and cystatins.

Growth factor - Epidermal growth factors

Buffers - Sialin, urea, carbonic acid bicarbonate.

Table 6: Composition of saliva

COMPONENT	FUNCTION	DIAGNOSTIC APPLICATIONS
Electrolytes Sodium, potassium, calcium, magnesium, bicarbonates and phosphates and hydrogen.[75]	➢ Remineralisation of tooth.[75] ➢ Stabilizes hydroxyapatite crystals in enamel.[75] ➢ Lowers the pH.	➢ Diagnostic marker of an active disease status in periodontal tissues.[76]
Immunoglobulins IgA[75] IgG IgM	➢ Neutralizes and excretes virus, bacteria and enzyme toxins. [75, 77] ➢ Inhibits adhesion of microorganisms.[77]	➢ Infectious diseases can be monitored by the presence of antibodies to the organisms.[78] ➢ IgA and IgG was more consistent in patients with less severe periodontitis and also is a better indicator of the local response than the serum titre.[79]
Proteins Gustin[75] Mucin[75]	➢ Growth and maturation of taste buds[75] ➢ Lubricates and protects the oral tissues, maintains the visco-elasticity[75], aids in mastication, lubrication and speech.	➢ Proteins are used as a biomarker for diagnosis of dental caries, periodontal diseases, infections and autoimmune diseases, genetic and psychological disorders and malignancies[78]
Enzymes Alpha amylase[75] Lysozyme, lactoferrin, Sialoperoxidases, mucin glycoprotein's, agglutinins, histatins, proline rich proteins, statherins and cystatins. [75]	➢ Alpha amylase initiates digestion of the starch.[75] ➢ Lysozyme hydrolyze the cellular wall of some bacteria. ➢ Histatins has Bactericidal and bacteriostatic effects. [75] ➢ Sialoperoxidases exhibits protection from the toxic and oxidant effects of hydrogen peroxide.[75]	➢ Measures the effectiveness of periodontal treatment by changes in levels of specific bacterial enzymes in whole saliva.[79]
Growth factor Epidermal growth factors	➢ It helps in wound contraction and tissue repair.	➢ Salivary basic fibroblast growth factor (bFGF) might be a potential biomarker for detecting OSCC development in recovering patients.[80]
Buffers Sialin, urea, carbonic acid bicarbonate.[75]	➢ It prevents colonization of potentially pathogenic microorganisms. ➢ Prevents enamel demineralization.[75]	➢ Quantification of buffer capacity in carious lesions by buffer capacity test.[79]

FORMS OF SALIVA

Saliva can be collected and used in different forms,

1. Resting or unstimulated whole saliva

Whole saliva is composed of secretions from the salivary glands, GCF, desquamated epithelial cells, microorganisms and leukocytes. It is easy to collect and is non-invasive. The unstimulated saliva is the mixture of secretions that enters the oral cavity in the absence of stimuli.

The methods for collecting are draining, spitting, suction and swab method. In spitting method, the ideal position to collect the saliva is when the patient is sitting in the upright position with head slightly tilted forward and eyes open and the individual is allowed to accumulate the saliva in the floor of the mouth and is made to spit out in a preweighed or graduated test tubes. This method can be used when the flow rate is minimal and it also minimises the evaporation of the saliva. The disadvantage is that it might have some stimulatory effect.[81]

2. Stimulated saliva

Stimulated saliva is secreted because of the masticatory and gustatory stimulations. The method for collecting is either stimulated physiologically by food intake or artificially stimulated by using paraffin wax, unflavoured chewing gum base, cotton puff, citric acids and sour candy drops. In salivary diagnostics, the unstimulated whole

saliva is more preferred than the stimulated saliva because of diluted concentrations of the biomarkers in the later.[81]

3. Glandular saliva

Glandular saliva is collected from the specific site of the oral cavity. The sites are parotid gland, submandibular gland and sublingual gland. The method followed is cannulation of the salivary ducts or by application of specific collecting devices to the opening of the ducts. The saliva from minor salivary gland like labial, buccal and palatine glands are collected by periopaper/ sailopaper absorbent method, pipette method or by a collecting prosthesis.[81]

SALIVA AS A BIOMARKER FOR CANCER DETECTION

"Salivaomics" encompasses a wide collection of technologies that is used to explore different types of genes and their methylation in saliva.[82] "Proteomics" is the technology used for the identification and quantification of overall protein content in a cell, tissue or an organism.[83] Salivary sample collection and processing is simple, non-invasive, cost-effective, precise and compatible. Recently, salivary diagnostics has drawn enormous attention for the detection of specific biomarkers. Molecules such as DNAs, RNAs, proteins, metabolites, and microbiota that are present in blood could be also present in saliva. Thus, saliva concentration changes of natural substances and large variety of molecules can be used to monitor the entire spectrum of health and the disease status of an individual.[82, 84]

Mallikarjuna Rao Koduru, Amitha Ramesh, Swathi Adapa et al (2017),[(85)]in a study has measured the salivary albumin levels in normal individuals (N=30), chronic periodontitis (N=30) and oral squamous cell carcinoma (N=30). It was found that the mean value of albumin in healthy individuals is 0.28 (SD is 0.19), chronic generalized periodontitis patients is 0.31 (SD is 0.19) and Oral squamous cell carcinoma is 0.82 (SD is 0.41) showing a significant rise in salivary levels of albumin in OSCC patients. Hence, the study concluded that salivary albumin levels could be used as a biomarker in Oral squamous cell carcinoma cases.

HIPPO PATHWAY COMPONENTS IN SALIVARY EPITHELIAL CELLS

Salivary glands are composed of branched epithelial ductal networks that terminate as acini that together produce, transport and secrete saliva. (Aleksander et al)

Large tumor supressor kinase 1 and 2 expression

Sung- Min Hwang, Mei Hong Jin, Yong Hwan Shin et al (2014),[(86)]in a study of salivary gland epithelial cells identified the expression of LPA (Lysophosphatidic acid) receptors and its role in Hippo signalling pathway in these salivary gland epithelial cells. It was found that LPA receptors are functionally expressed and plays a role in activating the Hippo pathway mediated by YAP/TAZ through Lats/Mob1 and RhoA/ROCK. Upregulation of YAP/TAZ dependent target genes such as CTGF, ANKRD1& CYR61 had been noted in LPA treated cells.

YAP/TAZ modulate the apoptotic pathway in epithelial cells treated with LPA which inturn upregulates capase 3 that bring about apoptosis.

Yes associated protein (YAP) expression

Aleksander D Szymaniak, Rongjuan Mi, Shannon E McCarthy et al (2017), [87]had studied the epithelial and the ductal patterning of sub mandibular salivary gland (SMG) in mouse using immunofluorescence. It was found that deletion of the Lats1 and Lats2 genes, which encode kinases that restrict nuclear Yap localization, results in defective morphogenesis, organ patterning and function of SMG. The study concluded that Lats1/2 mediated Yap is a critical regulator of ductal progenitor cell and is required for the morphogenesis and proper patterning of SMG epithelium and Yap localization by Lats1/2 is essential for the maturation of SMG ducts.

Table 7: Saliva as a biomarker for OSCC

AUTHOR	BIOMARKER	NUMBER OF SUBJECTS	SPECIMEN TYPE	METHOD	RESULT
Warnakulasuriya.S et al (2000) [88]	P53	26 per Specimen type ➢ OSCC subjects	➢ Saliva ➢ Serum ➢ Tissue	➢ ELISA ➢ IHC	➢ 64% had stabilized p53 protein in tissues. ➢ 27% had high levels of p53 antibody in serum. ➢ p53 antibodies were found only in the serum and saliva of patients showing p53 overexpression in their tumour tissues.
Dmitry A Ovchinnikov et al (2012) [89]	DAPK1, $p16^{INK4a}$, and RASSF1A genes	143 ➢ Head and neck squamous cell carcinoma (n = 143). ➢ Healthy non-smoker controls (n = 31).	➢ Saliva	➢ Methylation specific polymerase chain reaction	➢ DNA methylation of promoters genes in individuals with head and neck squamous cell carcinoma (HNSCC) with an overall accuracy of 81% .
Yun Feng et al (2019)[90]	Protease spectrum (35 proteases)	16 per group ➢ OSCC ➢ Oral benign masses ➢ Chronic periodontitis ➢ Normal individuals	➢ Saliva	➢ Human protease array kit ➢ ELISA ➢ Western blot ➢ Immunofluorescence	➢ OSCC patients contained increased number of proteases than those of other groups. ➢ The cut off values for MMP-1, cathepsin V, kallikrein-5 and ADAM9 were 119, 11.34, 9.29, 202.55 respectively.

3.5 ELISA

Molecular biology is highly promising and involves procedures that detects alterations at a molecular level much before they are seen under a microscope and before clinical changes occur. Molecular studies serve as basis for augment action of clinical assessment and classification of oral lesions as well as predict malignant potential of oral lesions, thus reducing the incidence and increasing the scope for early diagnosis and treatment of oral cancers.[33]

Enzyme immunoassay is a term used to describe the test that detects either antigen or antibodies or haptens in the specimen, by using enzyme subtrate system for detection.

Principle of ELISA

"Enzyme" is used to label one of the components of immunoassay (i.e antigen or antibody). "Immunosorbent" an absorbent material is used that specifically absorbs antigen or antibody present in the serum. The enzyme reacts with the substrate which in turn activates the chromogen to produce the colour. Sometimes, the substrate is chromogenic in nature. On reaction with enzyme, it changes its colour. The colour change is detected by spectrophotometer. The intensity of the colour is directly proportional to the amount of the detection molecule present in the test serum.[91]

Advantages of ELISA

ELISA is the method of choice for detection of antigen/antibody in serum in modern days. A large number of samples can be tested using the 96 well microtitre plate in big laboratories. It is economical and consumes 2-3 hours for performing the assay. It is the most sensitive immunoassay, hence is the choice for performing screening tests at blood banks and tertiary care sites. It is specficity used to be low. Specificity of the assay has increased with the use of more purified and synthetic antigens and monoclonal antibodies.[91]

Disadvantages of ELISA

In small laboratories having less sample load, ELISA is less preferred than rapid test as latter can be done on individual samples. It takes more time compared to rapid test which takes 10-20 minutes. It needs expensive equipments such as ELISA washer and reader.[91]

ELISA IN DETECTION OF CANCER

Mahnaz Saheb Jamee, Mohammad Eslami, Fazele Atarbashi Moghadam et al (2008),[92] in a study compared the concentration of tumor necrosis factor α (TNF α), interleukin 1α, 6, and 8 between oral squamous cell carcinoma patients (N=9) and healthy individuals (N=9) in saliva. The study concluded that the concentration of salivary interleukin 6 in oral squamous cell carcinoma patients was higher than control group and with statistically significant significance ($p < 0.05$). However, the concentration of salivary tumor necrosis factor α,

interleukin 1α and 8 in case group was higher than control group but was not statistically significant ($p > 0.05$).

T Shpitzer, Y Hamzany1, G Bahar1, R Feinmesser et al (2009),[93] in a study of 19 tongue cancer patients, measured the levels of eight salivary markers related to oxidative stress, DNA repair, carcinogenesis, metastasis and cellular proliferation and death. Five markers were increased in cancer patients by 39–246% which are carbonyls, lactate dehydrogenase, metalloproteinase-9 (MMP-9), Ki67 and Cyclin D1 (Cyc D1) at ($P \leqslant 0.01$). Three markers decreased by 16–29% which are 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) ($P \leqslant 0.01$).

Tao Jiang, Guoxia Liu, Lin wang et al (2015),[94] had studied the level of serum Gas6 and its clinical significance among OSCC patients and normal controls (N=45). The results showed serum Gas6 concentration significantly higher in OSCC patients than in controls ($P < 0.05$) and was also increased in late TNM stage (III, IV) and poorly differentiated tumors than those with early stage (I, II) ($P < 0.01$) and well differentiated tumors ($P < 0.01$). They concluded that serum Gas6 could be a candidate biomarker for diagnostic and prognostic use in OSCC patients.

ToshiroMoroishi, TomokoHayashi, Wei-WeiPan et al 2016,[95] have done a study to demonstrate that, in three different murine syngeneic tumor models (B16, SCC7 and 4T1), loss of the Hippo pathway kinases LATS1/2 (large tumor suppressor 1 and 2) in tumor cells inhibits tumor

growth. They have discovered an unexpected role of the Hippo pathway in suppressing anti-tumor immunity. Tumor regression by LATS1/2 deletion requires adaptive immune responses, and LATS1/2 deficiency enhances tumor vaccine efficacy. LATS1/2 dKO B16-OVA melanoma cells, and serum anti-OVA IgG concentrations were determined by ELISA 12 days after transplantation. Data are presented as means ± SEM; n = 4 mice for the uninjected group, n = 10 mice for the WT-injected group, and n = 10 mice for the LATS1/2 dKO-injected group. $^{***}p < 0.001$, one-way ANOVA test followed by Tukey's multiple comparison test.

Yun Feng, Qian Li, Jiao Chen et al (2019),[(90)]in a study analyzed the salivary protease spectrum among patients with oral squamous cell carcinoma (OSCC), oral benign masses, chronic periodontitis and healthy individuals using human protease array kits, ELISA, western blot and immunofluorescence. The proteases that were analysed by ELISA were ADAMTS13, cathepsin E, MMP-1, MMP-2, MMP-3, MMP-10, MMP-12 and MMP-13, ADAM9, cathepsin V, kallikrein 5 and kallikrein 7 among which MMP-1, MMP-2, MMP-10, MMP-12, cathepsin V, kallikrein 5 and ADAM9 were significantly increased in OSCC patients. The study concluded that cathepsin V, kallikrein 5 and ADAM9 may be useful biomarkers in the screening and diagnosis of OSCC.

MATERIALS AND METHODS

4.1 Study design:

A quantitative study was done to estimate the levels of LATS 1/2 in saliva by using ELISA test.

4.2 Study subjects:

Group I- Individuals with apparently healthy oral mucosa and no habits

Group II- Individual tobacco smokers with no PMD lesions

Group III-Individuals with tobacco associated potentially malignant disorder.

Group IV-Individuals with tobacco associated oral squamous cell carcinoma.

4.3 Sample size:

40 (10 subjects in each group).

Inclusion criteria:

- Current smoker
- Subjects with PMD.
- Subjects with OSCC.

Exclusion criteria:

- Subjects younger than 18 years of age group.
- Subjects undergone chemotherapy, radiotherapy and surgery.
- Subjects with xerostomia.
- Subjects with differently abled.

4.4 Study setting:

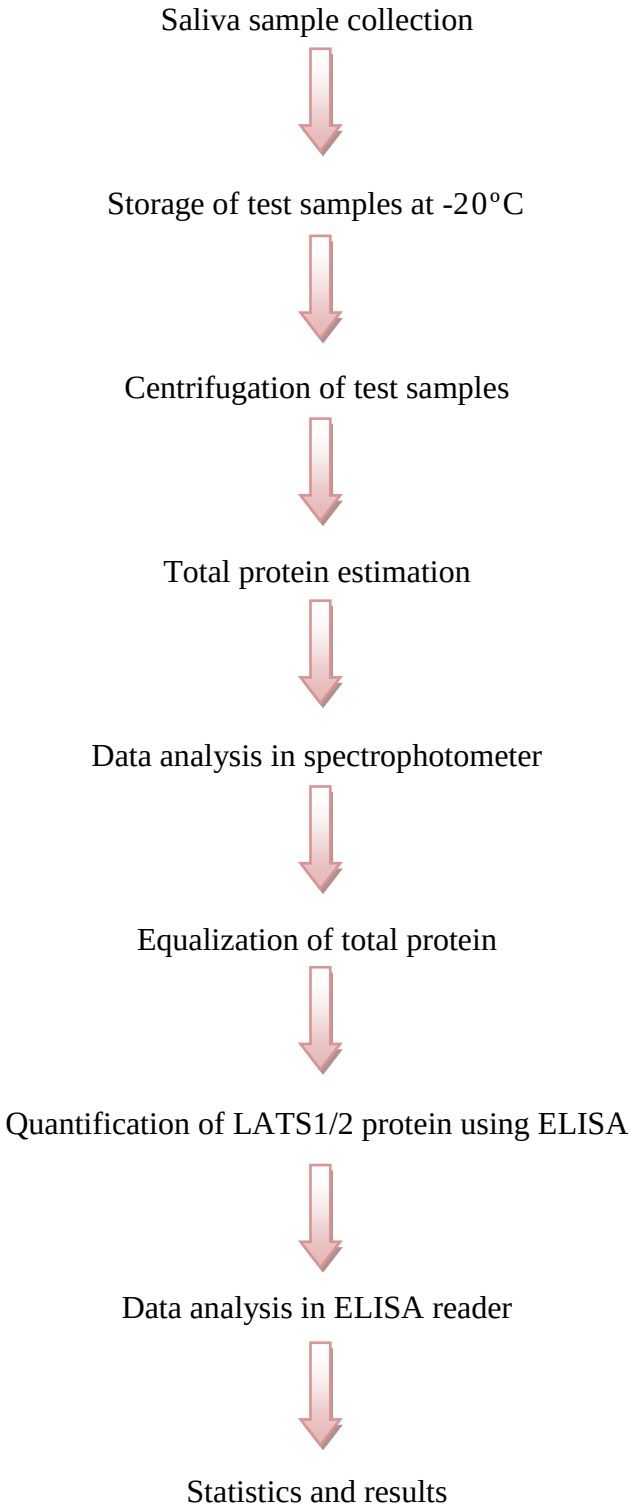
- Saliva samples was collected from patients attending the Chettinad Dental College and Research Institute.
- Study was conducted at Chettinad Dental College and Research Institute and Allied Health Sciences, Chettinad Academy of Research and Education, Chennai.
- Informed consent was obtained from the patient.
- Demographic details of the patient were recorded.
- A thorough oral examination was done and findings were recorded in a pre structured case sheet.
- The salivary samples were collected by oral rinse.
- The patient was asked to sit in the chair, and after spitting any residual saliva in the mouth, he or she was asked to spit in a sterile container every minute for 10 minutes to collect unstimulated whole saliva.
- The sample was immediately transported to the research laboratory at Allied Health Sciences, Chettinad Academy of Research and Education, Chennai and stored at -20ºC until the procedure was started.

4.5 Armamentarium required:

1. Mask and gloves
2. Working sheet /green cloth
3. Test samples(saliva samples)
4. -4ºC & -20ºC refrigerator

5. Eppendorf tubes
6. Eppendorf tube stand
7. Marker pen
8. Centrifugal spin
9. Tissue papers
10. 10µL,200µL and 1000µL micropipette
11. Micropipette tips
12. Micropipette tip holder
13. Reagents
14. Weighing machine
15. Spectrophotometer
16. LATS1/2 ELISA kit- Anti LATS1/2 antibody, Standard solution, Standard diluent, Streptavidin Horse radish peroxidase (HRP), Sealer, Wash buffer, Substrate A, Substrate B and Stop solution
17. Autoclave machine
18. Distilled water and Milli- Q water

Fig 9: Study procedure



4.6 STUDY PROCEDURE

Step 1 - Saliva sample collection

The patient was asked to sit on the dental chair and informed about the study following which informed consent was obtained. The case history and clinical examination of the patient was done. The patient was asked to spit out the residual saliva following which unstimulated whole saliva was collected into a sterile saliva sample container for every minute for 10 minutes. The corresponding saliva sample container was then labeled with their demographic details.

Step 2- Storage of test samples

The test samples after collection were stored immediately at -20ºC refrigerator/freezing unit to prevent the denaturation of protein thereby preserving it until the initiation of procedure.

Step 3- Centrifugation of test samples

The saliva samples were taken out from -20ºC refrigerator and thawed to room temperature. 500µl of the thawed test samples was transferred into a 2 ml capacity Eppendorf tube using 1000µl micropipette. The saliva samples (Group I, Group II, Group III and Group IV) were segregated and labeled by the alphabet T, symbolizing the test samples and numbered from T1 to T40. The test samples were centrifuged at 10,000 rpm for 2 minutes. Following the centrifugation, two components were seen, one is the supernatant and another is the pellet that is formed at the bottom. The supernatant contains the proteins and other enzymes and the pellet contains debris. The pellet

was discarded, the protein containing supernatant was transferred to separate Eppendorf tubes using a micropipette and was labeled with the corresponding test sample number. The tubes were stored at -20ºC until the subsequent procedure.

Step 4- Total protein estimation

The total protein estimation procedure was done in test sample (unknown protein) and standardized with a known protein- bovine serum albumin (BSA). The reagents required for total protein estimation were prepared as follows,

(i) Reagent preparation for unknown protein

For quantitative estimation, the method of Lowry et al (1951) was followed,

- **0.2(N) Sodium hydroxide (NaOH) solution**-1gm of NaOH dissolved in 250ml of Milli-Q water.
- **Alkaline copper sulphate solution-**

  Reagent A: 2% sodium carbonate (Na_2CO_3) dissolved in 0.1(N) NaOH solution

  Reagent B: 0.5% copper sulphate ($CuSO_4.5H_2O$) dissolved in 1% sodium potassium tartarate.

  50 ml of reagent A was mixed with 1 ml of reagent B to make the alkaline copper sulphate solution. This solution was freshly prepared before use.
- **Folin's reagent-** This reagent is either commercially available or freshly prepared before use. Folin's reagent is mixed with Milli-Q

water in 1:1 proportion and used for total protein estimation. Since it is a light sensitive chemical, it is freshly prepared and wrapped with tin foil paper.

(ii) Reagent preparation for known (standard) protein

- **Stock standard solution-** 50 mg of bovine serum albumin (BSA) was dissolved in Milli-Q water to a final volume of 50 ml.
- **Working standard solution-** 10 ml of the stock solution was diluted with Milli-Q water to make a total volume of 50 ml (Lowry et al 1951).

(iii) Procedure of total protein test

The Eppendorf tubes containing the test sample supernatant is taken out from -20ºC refrigerator and thawed to room temperature.

Known protein estimation- Six empty Eppendorf tubes were taken. One tube was labeled as 'B' denoting blank solution and five tubes were labeled as 'S' denoting standard solution. 500µl of alkaline copper sulphate solution was added to blank tube and grades of 10 µl, 20 µl, 30 µl, 40 µl and 50 µl of bovine serum albumin were added to S1, S2, S3, S4, S5 respectively. Positive reaction is denoted by colour change from clear to blue.

Unknown protein estimation- 40 empty Eppendorf tube one each for the test samples, labeled with the corresponding test sample number was taken. 500µl of alkaline copper sulphate solution was taken in the Eppendorf tube over which 20µl of test sample was added. This

mixture was kept for incubation at 37ºC for 10 minutes. After 10 minutes, 50µl of Folin's reagent was added and incubated under dark for 30 minutes. A blue color change was noted which is variable depending upon the concentrations of total protein in the sample. The samples were incubated for 1 hour in dark. Reanalysis or duplication of the test was done for each sample to avoid human/manual error.

(iv)Data analysis- The test samples were analyzed in UV spectrophotometer. The absorbance value (OD) was measured at 650nm and the readings were plotted to obtain a concentration curve for both standard and test sample. The OD values of all test samples were used for calculation of total protein concentration using Microsoft Excel version 2007.

Step 5: Equalization of total protein

All test samples had varied protein concentrations which varies from one individual to another. The calculated protein concentration were converted into volume (µl) to equalize the test sample concentration as 1 microgram of protein in all samples. After calculation, the obtained volume (µl) was used to quantify LATS1/2 by ELISA.

Step 6: Quantification of LATS1/2 protein

Enzyme-Linked Immunosorbent Assay (ELISA) test was used to quantify the large tumor suppressor kinase 1 and 2 (LATS1/2). Human LATS1/2 antibody pre-coated 96 well ELISA plate was used. The ELISA kit was kept at -4ºC before use.

REAGENT PREPARATION FOR ELISA

Standard solution for LATS1 - The standard solution containing the biotinylated LATS1 antibody of 9.6ng/ml concentration and standard diluent of 0 ng/L was provided in the kit. The standard solution was allowed to thaw for 15 minutes with gentle agitation prior to making dilutions. 120µl of the standard (9.6ng/ml) was diluted with 120µl of standard diluent to generate a 4.8 ng/ml standard stock solution. Duplicate standard points (Table 8) were prepared by serially diluting the standard stock solution (4.8ng/ml) in 1:2 ratio with standard diluent to produce 2.4ng/ml, 1.2ng/ml, 0.6ng/ml and 0.3ng/ml solutions. The descending concentrations of LATS1/2 standard were labeled as S5, S4, S3, S2, S1 respectively.

Table 8: Standard dilution for LATS1*

Conc. of standard	Standard number(No.)	Serial dilutions of standard
4.8ng/ml	Standard No.5	120µl Original standard + 120µl Standard Diluent
2.4ng/ml	Standard No.4	120µl Original standard No.5 + 120µl Standard Diluent
1.2ng/ml	Standard No.3	120µl Original standard No.4 + 120µl Standard Diluent
0.6ng/ml	Standard No.2	120µl Original standard No.3 + 120µl Standard Diluent
0.3ng/ml	Standard No.1	120µl Original standard No.2 + 120µl Standard Diluent

***User manual- Bioassay technology laboratory**

Standard solution for LATS2- The standard solution containing the biotinylated LATS2 antibody of 3,200ng/L concentration and standard diluent of 0 ng/L was provided in the kit. The standard solution was allowed to thaw for 15 minutes with gentle agitation prior to making dilutions. 120µl of the standard (3200ng/L) was diluted with 120µl of standard diluent to generate a 1600ng/L standard stock solution.

Duplicate standard points (Table 9) were prepared by serially diluting the standard stock solution (1600ng/L) in 1:2 ratio with standard diluent to produce 800ng/L, 400ng/L, 200ng/L and 100ng/L solutions. The descending concentrations of LATS2 standard were labeled as S5, S4, S3, S2, S1 respectively.

Table 9: Standard dilution for LATS2*

Conc. of standard	Standard number(No.)	Serial dilutions of standard
1600ng/ml	Standard No.5	120µl Original standard + 120µl Standard Diluent
800ng/ml	Standard No.4	120µl Original standard No.5 + 120µl Standard Diluent
400ng/ml	Standard No.3	120µl Original standard No.4 + 120µl Standard Diluent
200ng/ml	Standard No.2	120µl Original standard No.3 + 120µl Standard Diluent
100ng/ml	Standard No.1	120µl Original standard No.2 + 120µl Standard Diluent

***User manual- Bioassay technology laboratory**

Distilled Milli-Q water- The Milli-Q water was taken in a air tight glass container and sterilized in an autoclave at 121ºC for 15 -20 minutes and cooled to room temperature.

Wash buffer- Commercially available in the ELISA kit.2ml of wash buffer was mixed with 48ml of distilled water to make a total of 50ml.

PROCEDURE:

Standard solution for LATS1

In the 96 well precoated ELISA plate for LATS1, five wells were used as standard wells. 50µl of standard solutions S1, S2, S3, S4, S5 prepared for LATS1 along with 50µl of Streptavidin horse radish peroxidase (HRP) were added to the corresponding five standard wells for the assay of LATS1.

Test sample for LATS1

All the test samples and reagents were kept at room temperature for 30 minutes before the assay. The number of test wells required for the assay were determined and inserted into the frame. A total of 80 wells were utilized in which 40 test samples were analyzed and the same 40 samples were re-analyzed to carry out the process of duplication. Duplication is usually done to avoid human/manual error. 40µl of test sample, 10µl of anti-LATS1 antibody and 50µl Streptavidin-HRP were added to all the wells respectively. The plate was covered with a transparent sealer, which was provided along with the kit. The sample and the reagents were gently mixed by tapping on a flat surface. All the 80 wells were incubated at 37ºC for 60 minutes. After 60 minutes, a colour change from clear to blue was noted. The sealer was removed. The wells were soaked with 0.35ml of wash buffer for 1 minute, the same being repeated 5 times. This is done to wash the excess and remaining unbounded antigens in the test sample. The ELISA plate was blotted with tissue paper to remove residual wash buffer. After blotting thoroughly, 50µl substrate solution A followed by 50µl substrate solution B was added to each well. The wells were covered with tin foil and incubated at 37ºC for 10 minutes. After incubation, 50µl of stop solution was added to each well to stop the reaction between test sample and reagents following which the colour changes from blue to yellow indicating the presence of LATS1 antigen in the sample.

Standard solution for LATS2

In the 96 well precoated ELISA plate for LATS2, five wells were used as standard wells. 50µl of standard solutions S1, S2, S3, S4, S5 prepared for LATS2 along with 50µl of Streptavidin horse radish peroxidase (HRP) were added to the corresponding five standard wells for the assay of LATS2.

Test sample for LATS2

All the test samples and reagents were kept at room temperature for 30 minutes before the assay. The number of test wells required for the assay were determined and inserted into the frame. A total of 80 wells were utilized in which 40 test samples were analyzed and the same 40 samples were re-analyzed to carry out the process of duplication. Duplication is usually done to avoid human/manual error. 40µl of test sample, 10µl of anti-LATS2 antibody and 50µl Streptavidin-HRP were added to all the wells respectively. The plate was covered with a transparent sealer, which was provided along with the kit. The sample and the reagents were gently mixed by tapping on a flat surface. All the 80 wells were incubated at 37ºC for 60 minutes. After 60 minutes, a colour change from clear to blue was noted. The sealer was removed. The wells were soaked with 0.35ml of wash buffer for 1 minute, the same being repeated 5 times. This is done to wash the excess and remaining unbounded antigens in the test sample

The ELISA plate was blotted with tissue paper to remove residual wash buffer. After blotting thoroughly, 50µl substrate solution

A followed by 50µl substrate solution B was added to each well. The wells were covered with tin foil and incubated at 37°C for 10 minutes. After incubation, 50µl of stop solution was added to each well to stop the reaction between test sample and reagents following which the colour changes from blue to yellow indicating the presence of LATS2 antigen in the sample.

Step 7: Data analysis in ELISA reader

The optical density (OD value) of each well was quantified immediately within 10 minutes after adding the stop solution. The OD values were determined using an ELISA reader at 450 nm. The digital data sheet containing the LATS1/2 protein levels were obtained immediately.

Step 8: Statistics and results

All the values and parameters were analyzed by one-way ANOVA test and independent sample t-test.

RESULTS

A total number of 40 cases comprising 10 normal controls (Group I), 10 subjects of smokers without lesion (Group II), 10 subjects with potentially malignant disorder (Group III) and 10 subjects with oral squamous cell carcinoma (Group IV) were studied.

Age distribution among subjects

The age range of the study subjects were between 22 years and 63 years. The age distribution was 20-40 years (57.5%), 41-60 years (37.5%) and above 60 years (5%). (Graph 1)

Gender distribution among subjects

The gender distribution among the subjects were 26 males (65 %) and 14 females (35 %). (Graph 2)

Expression of LATS1 and LATS2 among the study group

The results were statistically obtained using one-way ANOVA test which showed variable expression between LATS1 and LATS2 in the respective study groups and between the study groups. Group I (Normal control) had a mean LATS1 and LATS2 value of 2.7047ng/ml and 9.9093ng/ml respectively, Group II (Smokers without lesion) had a mean LATS1 and LATS2 value of 3.2828 ng/ml and 7.6075ng/ml respectively, Group III (Subjects with PMD) had a mean LATS1 and LATS2 value of 4.5442ng/ml and 10.8238ng/ml respectively and in Group IV (Subjects with OSCC) the mean LATS1 and LATS2 value was 5.0524ng/ml and 3.0849ng/ml respectively.

The mean expression value of LATS1 showed a gradual rise from the Group I to Group IV which was statistically significant at p-value 0.001. (Table 10 & 12, Graph 3 & 4)

The mean expression of LATS2 showed a gradual decline in value from Group I to Group IV that was statistically significant at p-value 0.035, although the mean value was highest in Group III. (Table 11 & 12, Graph 3 & 4)

Cytological grade distribution among the study subjects

The cytological grades among the total subjects were class I - 42.5% (17 subjects), class II - 30% (12 subjects), class III - 15% (6 subjects) and class IV -12.5% (5 subjects).

Comparison of LATS1expression to cytology grades

The results were statistically obtained using one-way ANOVA test which revealed that the mean value of LATS1 among Class I subjects were 3.2635ng/ml, Class II was 3.9481ng/ml, Class III was 4.5209ng/ml and class IV was 5.1717ng/ml. The results infer that there is a progressive rise in the expression of LATS1 from Class I to Class IV cytology grades and the association between the LATS1 levels and cytology grades were significant at p-value 0.006. (Table 13, Graph 5 & 6)

Comparison of LATS2 expression to cytology grades

The results were statistically obtained using one-way ANOVA test which revealed that the mean value of LATS2 among subjects with

Class I cytology was 10.7037ng/ml, Class II was 7.6853ng/ml, Class III was 5.1698ng/ml and class IV was 2.5950ng/ml. The results infer a gradual decline in the expression of LATS2 from class I to class IV, although its association with the cytology grades were statistically insignificant at p-value 0.182. (Table 13, Graph 5 & 6)

Histopathological grade distribution among OSCC cases (Group IV)

The histopathological grade among the group IV subjects were moderately differentiated squamous cell carcinoma 40% (4 subjects) and well differentiated squamous cell carcinoma 60% (6 subjects),

Comparison of LATS1 expression to histopathological grades

The results were statistically obtained using Independent Samples t-test which revealed the mean value of LATS1 among moderately differentiated OSCC as 5.1974ng/ml and well differentiated OSCC as 4.9557ng/ml. The results infer that there is a decrease in the expression of LATS1 from moderately differentiated to well differentiated oral squamous cell carcinoma (p-value 0.756). (Table 14, Graph 7)

Comparison of LATS2 expression to histopathological grades

The results were statistically obtained using Independent Samples t-test which revealed that the mean value of LATS2among moderately differentiated OSCC as 4.5287ng/ml and well differentiated OSCC as 2.1223ng/ml. The results infer a decline in the expression of LATS2 from moderately differentiated to well differentiated OSCC similar to that found in LATS1. The association between the LATS2 levels and histopathology grades had a p-value 0.129. (Table 14, Graph 7)

TABLES

Table 10: LATS1 expression (ng/ml) in all subjects group wise (graph 3)

GROUP I	GROUP II	GROUP III	GROUP IV
2.699625	4.362462	4.919311	3.611127
3.230858	3.629282	4.439063	5.714418
3.636494	2.791149	3.661862	3.407935
0.940043	2.811294	5.489838	6.366521
2.795874	3.477821	4.959352	4.691995
3.055024	4.047851	4.218711	6.207847
2.655356	3.222153	4.77357	3.981696
1.72371	4.054069	4.574855	6.214562
3.021449	2.523045	4.155291	5.23044
3.289055	1.908746	4.250048	5.097383
Mean=2.7047ng/ml	**Mean=3.2828ng/ml**	**Mean=4.5442ng/ml**	**Mean=5.0524ng/ml**

Table 11: LATS2 expression (ng/ml) in all subjects group wise (graph 3)

GROUP I	GROUP II	GROUP III	GROUP IV
6.414822	8.010643	1.534059	3.271901
7.340348	11.87379	6.217308	7.438111
8.236759	6.391675	6.007483	4.398682
10.40984	3.681007	15.01698	6.551566
8.865854	7.99326	6.056815	1.445092
14.69352	5.875931	27.90364	1.545692
7.810303	9.426108	1.175374	3.582742
10.79531	6.819182	8.751767	0.788612
15.32986	7.759533	26.51884	0.973257
9.196038	6.411472	9.056118	0.85336
Mean=9.9093ng/ml	**Mean=7.6075ng/ml**	**Mean=10.8238ng/ml**	**Mean=3.0849ng/ml**

Table 12: Expression of mean LATS1 and LATS2 in all study groups

	Group								One way ANOVA	
	GROUP I Healthy control		GROUP II Smoker without lesion		GROUP III Subjects with PMD		GROUP IV Subjects with Squamous Cell Carcinoma			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F-Value	P Value
LATS 1	2.7047	.8025	3.2828	.7772	4.5442	.5158	5.0524	1.1026	17.350	**0.001***
LATS 2	9.9093	3.4385	7.6075	2.6367	10.8238	9.4844	3.0849	2.4209	3.281	**0.035***

***p-value statistically significant at <0.05**

Table 13: Expression of mean LATS1 and LATS2 associated with cytology grades

	Cytology Grade								One way ANOVA	
	Class I		Class II		Class III		Class IV			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F-Value	P-Value
LATS 1	3.2635	1.1266	3.9481	.8866	4.5209	.9847	5.1717	1.4983	4.857	**.006**
LATS 2	10.7037	6.7512	7.6853	7.5405	5.1698	3.9550	3.8584	2.5950	1.738	.182

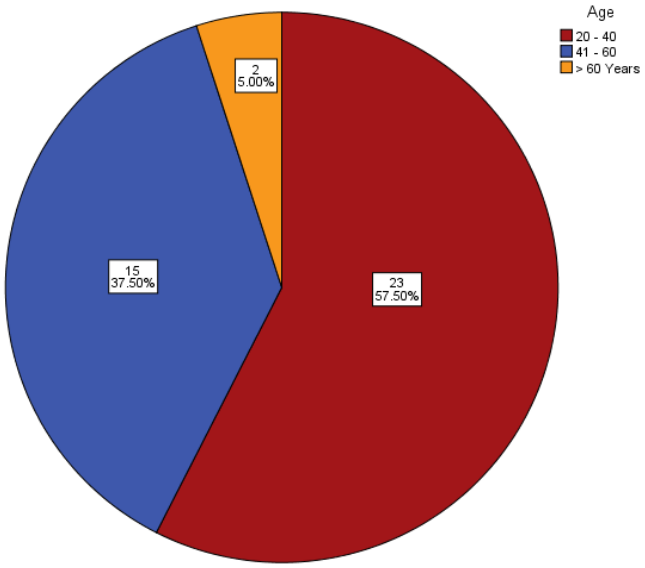
***p-value statistically significant at <0.05**

Table 14: Expression of mean LATS1 and LATS2 associated with histopathology grades in Group IV

	Histopathology Grade				Independent Samples t-test	
	Moderately differentiated		Well differentiated			
	Mean	SD	Mean	SD	t-Value	P-Value
LATS 1	5.1974	1.1776	4.9557	1.1526	.322	**.756**
LATS 2	4.5287	3.0356	2.1223	1.4970	1.692	**.129**

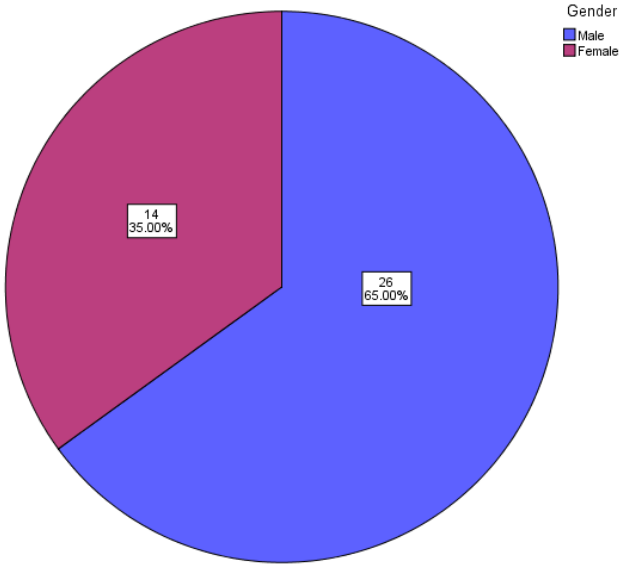
GRAPHS

AGE DISTRIBUTION



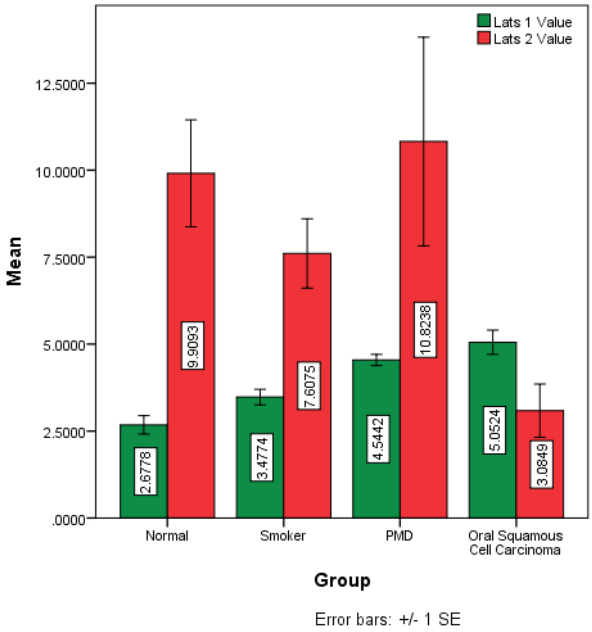
Graph 1: Age range of the study subjects distribution in percentage

GENDER



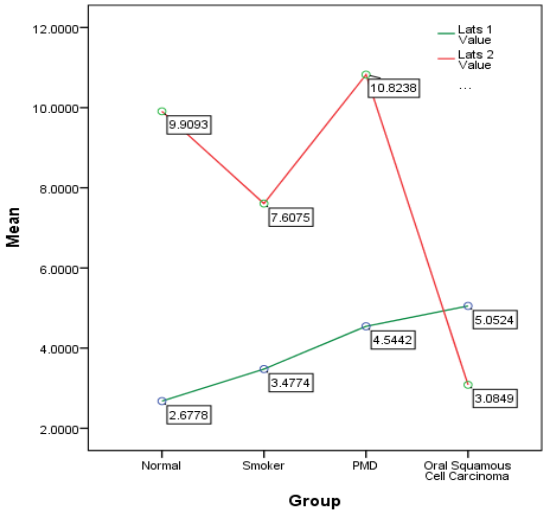
Graph 2: Gender distribution in the study

MEAN EXPRESSION OFLATS1 AND LATS2 IN STUDY GROUPS



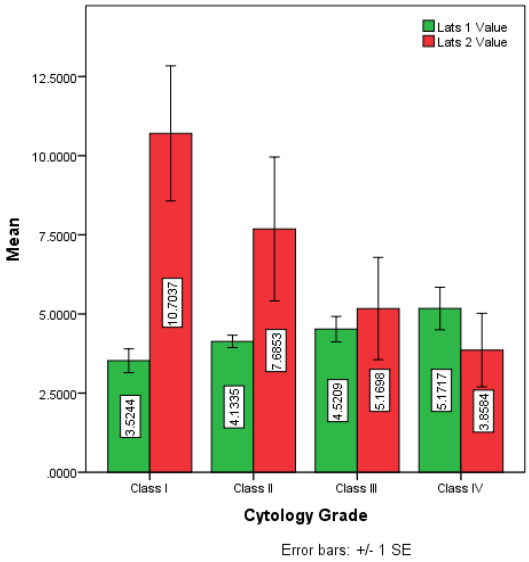
Graph 3: LATS1 and LATS2 expression in normal control, smokers without lesion, subjects with PMD and OSCC

COMPARISON OF LATS1 AND LATS2 AMONG THE STUDY GROUPS



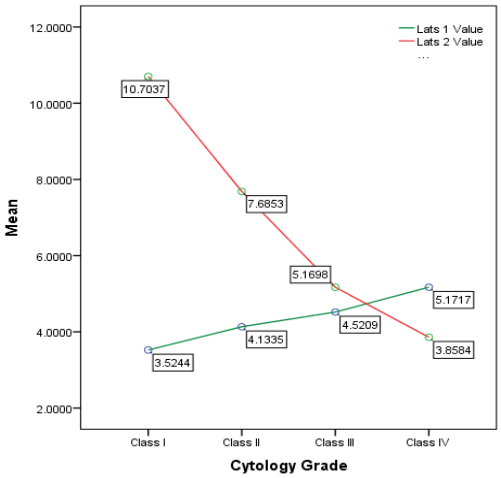
Graph 4: Scattered line diagram showing the gradual rise in the mean LATS1 expression and decline in the mean LATS2 expression between the study groups (ng/ml)

COMPARISON OF LATS1 AND LATS2EXPRESSION WITH CYTOLOGY GRADES



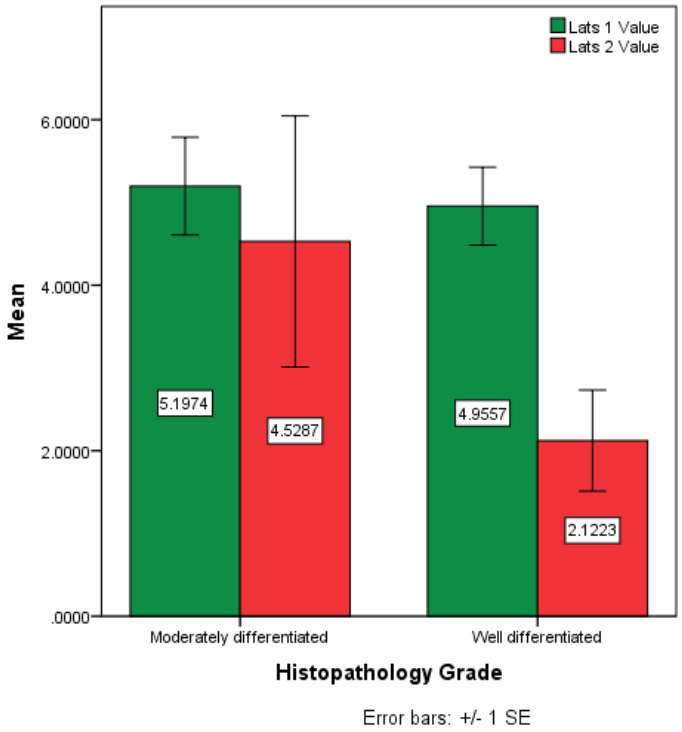
Graph 5: LATS1 and LATS2 expression in Class I to Class IV cytology grades

COMPARISON OF LATS1 AND LATS2 EXPRESSION WITH CYTOLOGY GRADES



Graph 6: Scattered line diagram showing the gradual rise in the mean LATS1 expression and decline in the mean LATS2 expression from Class I to Class IV cytology grades (ng/ml)

COMPARISON OF LATS1 AND LATS2 EXPRESSION WITH HISTOPATHOLOGICAL GRADING IN OSCC



Graph 7: Level of mean LATS1 and LATS2 expression compared to histopathology grades in OSCC (Group IV)

DISCUSSION

7.1 DISCUSSION OF RESULTS

Large Tumor Suppressor (LATS) family of human tumor suppressors (LATS1 and LATS2) is a governor of cellular homeostasis. Due to their high degree of homology and functional overlap, LATS1 and LATS2 comprise a new tumor suppressor family. LATS1 and LATS2 kinases share approximately 50% and 85% amino acid sequence identity between the full proteins and the kinase domains.[96]LATS1 and LATS2 are important signalling molecules with broad regulatory functions such as transcriptional regulation and maintenance of genetic stability in addition to more specific roles in modulation of cell cycle checkpoints, induction of apoptosis and inhibition of cell migration. LATS possess multiple functions like cell proliferation, cell death and cell migration, as well as broad governing roles such as transcriptional regulation and maintenance of genetic stability.[97]

In the present study the expression of LATS1 and LATS2 was quantified and compared with different groups. The study comprised of a total number of 40 cases comprising 10 normal individuals (Group I), 10 subjects of smokers without lesion (Group II), 10 subjects with potentially malignant disorder (Group III) and 10 subjects with oral squamous cell carcinoma (Group IV).

The values obtained from different groups were tabulated and analysed statistically. LATS1 mean value and LATS2 mean values were also compared with cytology and histopathology grades.

Comparison of LATS1 and LATS2 expression in study groups

In the present study, differing expression pattern of mean LATS1 and mean LATS2 was found among the study groups. A gradual rise in the expression of LATS1was evident from Group I to Group IV whereas LATS2 showed a gradual decline in expression from Group I to Group IV although the mean value in the Group III was higher than the other groups. (Table 10, 11, 12 &Graph 3, 4)

Stacy Visser et al[98]found varying level of LATS1 and LATS2 in normal tissues. LATS1 and LATS2 protein levels can be modulated at several levels in response to different stimuli and also have different expression patterns during development. Prominent expression of LATS1 was found in tissues of ectodermal origin such as the neural tube and neuroepithelium, whereas highest LATS2 expression was found in tissues of mesodermal origin including the cardiac outflow tract and heart. The differing levels of expression in both developing and adult tissues suggests that LATS1 and LATS2 may possess distinct roles.

The results of the present study revealed differing levels of LATS1 and LATS2 which was in accordance with **Bing Xu, Duoxiang Sun, Zhihua Wang et al (2015),**[99]whose study to explore the expression levels of LATS family members in ovarian tumors using normal ovaries, fallopian tubes and endometrium as controls. Their study revealed LATS1 expression in normal ovarian tissues, but LATS2 was undetectable. Both LATS1 and LATS2 were highly expressed in

the fallopian tube tissues. Interestingly, LATS1 showed 34% high expression in ovarian serous cystadenomas and 28% high expression in ovarian mucinous carcinoma and 13% high expression in ovarian serous carcinomas than LATS2.

The decreased LATS1 expression and increased LATS2 expression in normal controls (Group I) when compared to expression among the subjects with OSCC (Group IV) in the present study may be considered as an indicator during the disease progression.

Expression of LATS1 in the study groups

In the present study, a gradual rise in LATS1 expression was noted from Group I to Group IV. The LATS1 level in oral squamous cell carcinoma subjects (Group IV) was high when compared with normal controls (Group I), smokers without lesion (Group II) and potentially malignant disorder (Group III).

Masami Nozaki, Norikazu Yabuta et al 2019,[(44)]found that LATS1/2 were highly expressed in the oral squamous cell carcinoma line SAS. They also demonstrated that LATS1/2 are essential for self-renewal of OSCC cells with CSC properties, probably via activation of SNAIL and TAZ. These results suggest that, in SAS cells, LATS1/2 are potentially over expressed and probably activated.

Fengyuan Tang1, Ruize Gao1, Beena Jeevan-Raj1, Christof B et al,[(96)]found that LATS1 messenger RNA levels were found to be significantly higher, whereas LATS2 mRNA levels were rather lower in

hepatocellular carcinoma patient samples. High levels of LATS1 mRNA showed a pro-tumorigenic role of LATS1 and also correlated with poor overall and disease-free survival of hepatocellular carcinoma patients.

Jicheng Wu et al,[100] concluded that cytoplasmic LATS1 expression was stronger in primary cancer than dysplasia and normal epithelium ($P < 0.05$). Cytoplasmic LATS1 expression in normal squamous epithelium was 9.7% (11/113), in dysplasia 15.6% (15/99) and in primary cancer 62.5% (318/509).

The present study also showed a gradual rise in LATS1 expression in the subjects with OSCC when compared to the normal controls (Group I), smokers without lesion (Group II) and potentially malignant disorders (Group III). Also, LATS1 expression in Group II was between Group I and Group III indicating the possible association of tobacco in the disease process.

In the present study, LATS1 expression was also correlated with cytology and histopathology grades. Literature search reveals no similar comparisons done before.

In the present study a gradual rise in LATS1 expression was evident from Class I to Class IV cytology grades. Comparison of histopathological grades in the Group IV subjects showed a gradual rise in LATS1 expression from well differentiated to moderately differentiated OSCC.

Although LATS1 is tumor suppressor and its down regulation should be evident in oral squamous cell carcinoma, contrarily, the present study results were in accordance to many authors who had reported its overexpression in OSCC.

Expression of LATS2 in study groups

In the present study a gradual decline in LATS2 expression was noted from Group I to Group II followed by a hike in Group III. LATS2 expression was comparatively less in Group IV than Group I and Group III.

The present study results in Group IV was in accordance with the study done by **Yue ZW et al 2017,**[41]which concluded as exhibition of 68% promotor methylation of LATS2 gene in OSCC tissues indicating the significant role of LATS2 in oral carcinogenesis.

Feng Yao & Hongcheng Liu & Zhigang Li et al 2014,[101] found that the expression level of LATS2 was decreased in non-small cell lung carcinoma (NSCLC) tissues. Moreover, forced expression of LATS2 in NSCLC cells inhibited cell growth and migration, while knockdown of the expression of LATS2 promoted the tumorigenicity of NSCLC cells. Their study suggested that down-regulation of LATS2 was very important in the progression of NSCLC, and restoring the function of LATS2 might be a promising therapeutic strategy for NSCLC.

Contrary to the findings of the present study, **Wei-Wei et al 2018,**[102]have found that deletion of LATS1/2 inhibited the growth of murine MC38 colon cancer cells (N=8), especially under detachment conditions. They have also concluded that LATS1/2 inhibition can be used in therapeutics of oral squamous cell carcinoma.

The present study showed decreased expression of LATS2 in Oral squamous cell carcinoma subjects. LATS2 can be used as a biomarker in OSCC for predicting the treatment outcome.

In the present study, LATS2 expression were also correlated with cytology and histopathology grades. Among the study subjects, a gradual decline in LATS2 expression was evident from Class I to Class IV cytology grades. In Group IV a gradual rise in LATS2 expression was evident from well differentiated to moderately differentiated histopathology grades.

The present study results was in accordance with **Xiaoming Dai**[1]**, Huan Liu, Shuying Shen et al**[103] found that Yes associated protein (YAP-oncogene) activity strongly activates its upstream kinase inhibitors LATS1/2. Physiological activation of YAP is followed by a negative feedback activation of Lats2 transcription and Lats2 kinase activity, which normalizes YAP activity.

They also examined this possibility in vivo by expressing YAP-5SA transposon plasmids in mouse liver using hydrodynamic injection. Remarkably, while Lats1/2 phosphorylation was mildly induced on

post-injection day 1, strong on day 5, as accompanied by elevated Lats2 and NF2 protein levels. However, as YAP-5SA is insensitive to the Hippo pathway kinases, tumorigenesis occurred within 3 months regardless of Hippo pathway activation.

Though the overall expression of LATS2 is decreased in OSCC, the moderately differentiated OSCC showed increased LATS2 expression than well differentiated OSCC. This indicates that YAP might be actively/increasingly present towards severe grades of OSCC thereby leading to increased expression of LATS2 in an attempt to normalize the oncogenic activity of YAP. The cause may be attributed to the negative feedback mechanism or might be due to the insensitivity of YAP to LATS2 kinases indicating the progression of the OSCC.

7.2 UNIQUENESS OF THE STUDY

1. The present study is one among the few studies done in both the large tumor suppressor family members, LATS1 and LATS2.
2. The study was first of its kind to the best of our knowledge where the expression of LATS1 and LATS2 was analysed in subjects of varied groups comprising normal individuals, smokers without lesion, potentially malignant disorders, oral squamous cell carcinoma.
3. Literature search confirms that the present study to the best of our knowledge is the first to use ELISA on saliva sample in an attempt analyse the expression of LATS1 and LATS2.

4. The expression of LATS1 and LATS2 were also compared with the cytology and histopathology grades of the subjects which had not been performed in oral cancer and oral potentially malignant disorders.
5. The study also compared the association of tobacco in potentially malignant disorder and oral squamous cell carcinoma which has not been conducted previously.

7.3 LIMITATIONS OF THE STUDY

1. Minimal number of samples were included in the study due to the expenses involved in the procedures.
2. Age and gender distribution were uneven due to the variable predilection of the disease process among the present study group.
3. Comparison of the LATS1 and LATS2 to the histopathological evaluation among Group I, Group II and Group III consisting normal individuals, smokers without lesion and subjects with potentially malignant disorders respectively was not possible due to the invasive procedure involved.
4. Comparison of LATS expression with survival rate and long term was not done due to the time constraint.

SUMMARY AND CONCLUSION

Large tumor suppressor family comprising of large tumor suppressor kinases 1 and 2 (LATS1 & LATS2) are the key tumor suppressors of the cell regulatory pathway called as Hippo signalling pathway.

Though saliva is not widely used in cancer diagnosis and prognostic evaluation, the present study concludes that LATS1 and LATS2 proteins are sufficiently expressed in saliva. Its expression in saliva can be valuable in observing the disease transformation from a potential malignant disorder to carcinoma. Hence the expression of LATS1 and LATS2 can be used to monitor the disease process.

Further studies with larger sample size and diverse clinico-pathological correlation will aid in arriving at a standard baseline value of LATS expression. This may help in the early prediction of oral squamous cell carcinoma at its precancerous stage. Future research can be carried out in saliva to study the expression and the role of other components involved in Hippo signalling pathway to understand its action in oral carcinogenesis.

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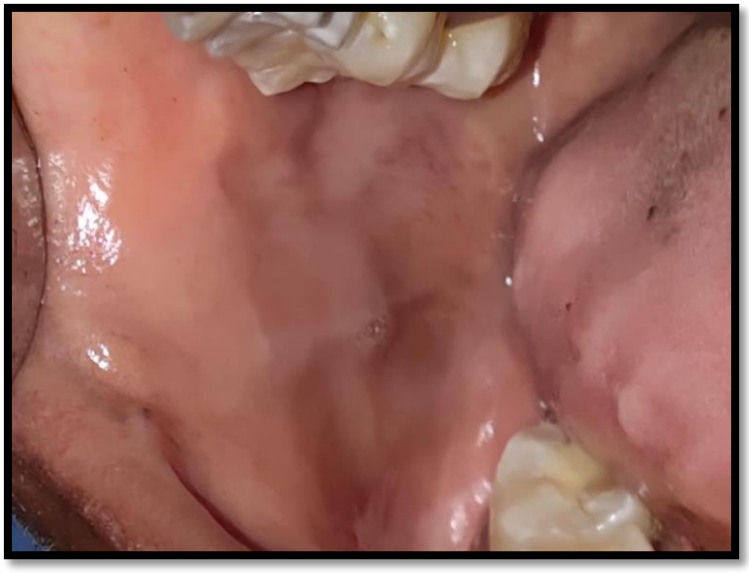
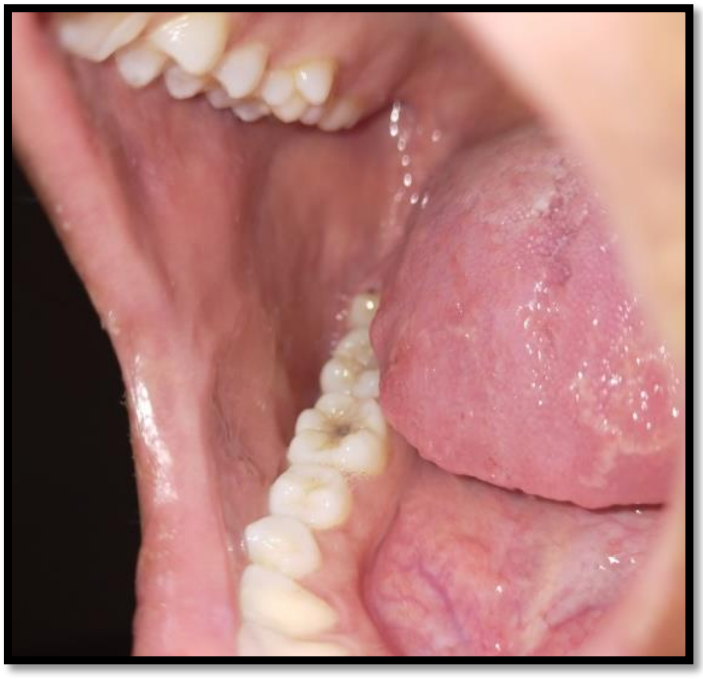
GROUP I- NORMAL CONTROL

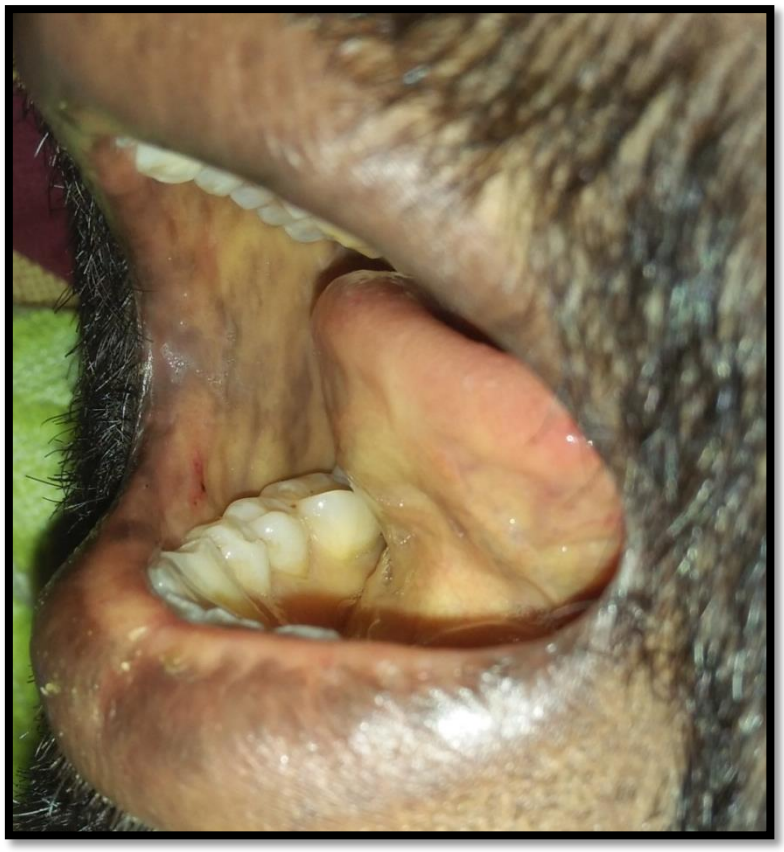


Photograph 1: Apparently normal buccal mucosa

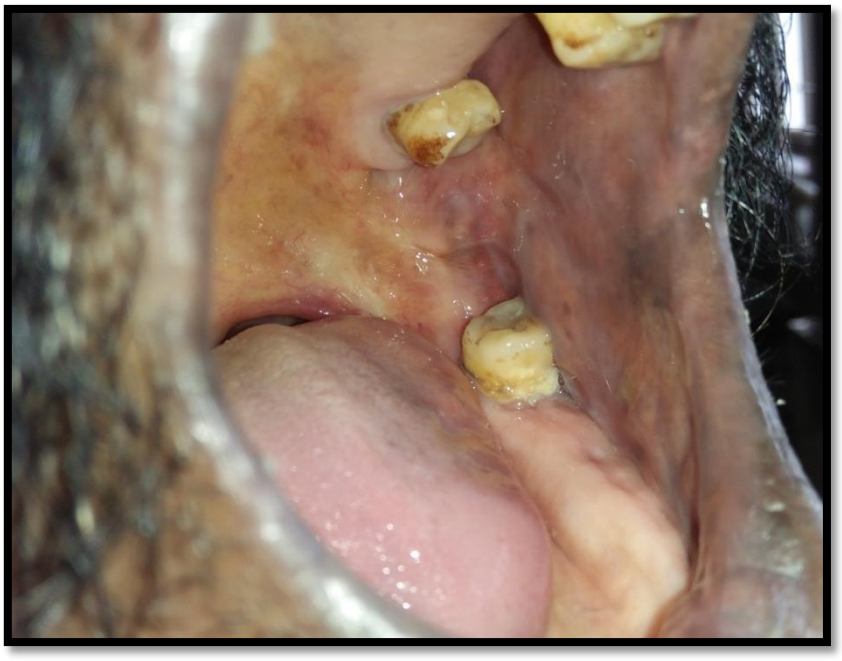


Photograph 2: Apparently normal buccal mucosa

GROUP II- SMOKERS WITH NO LESION

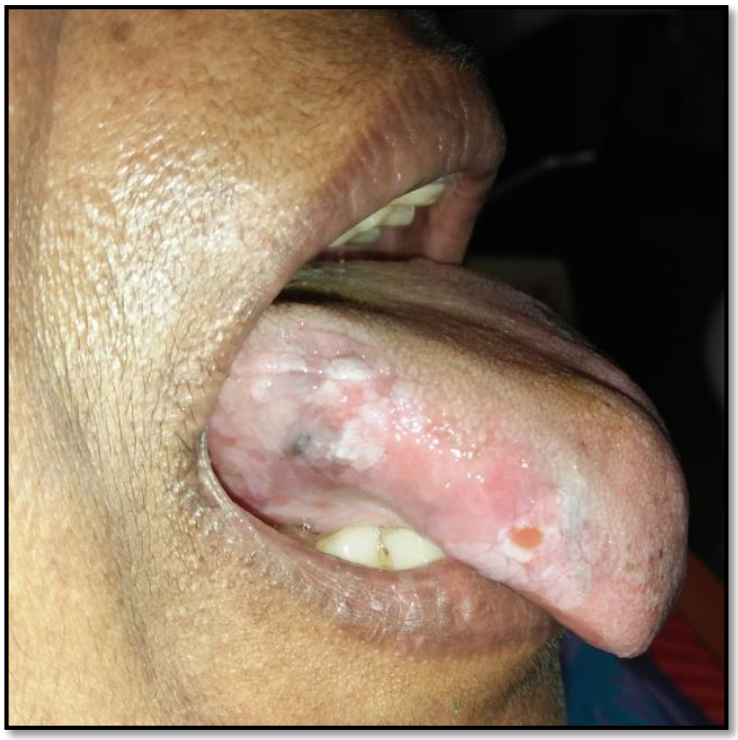


Photograph 3: Buccal mucosa showing pigmentation

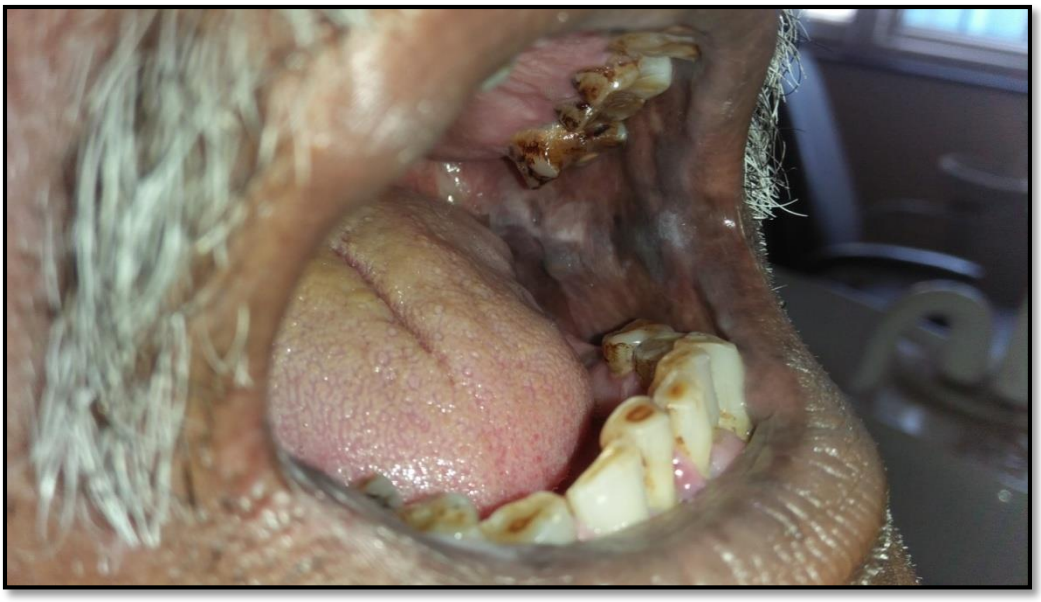


Photograph 4: Buccal mucosa showing pigmentation

GROUP III- TOBACCO ASSOCIATED POTENTIALLY MALIGNANT DISORDER

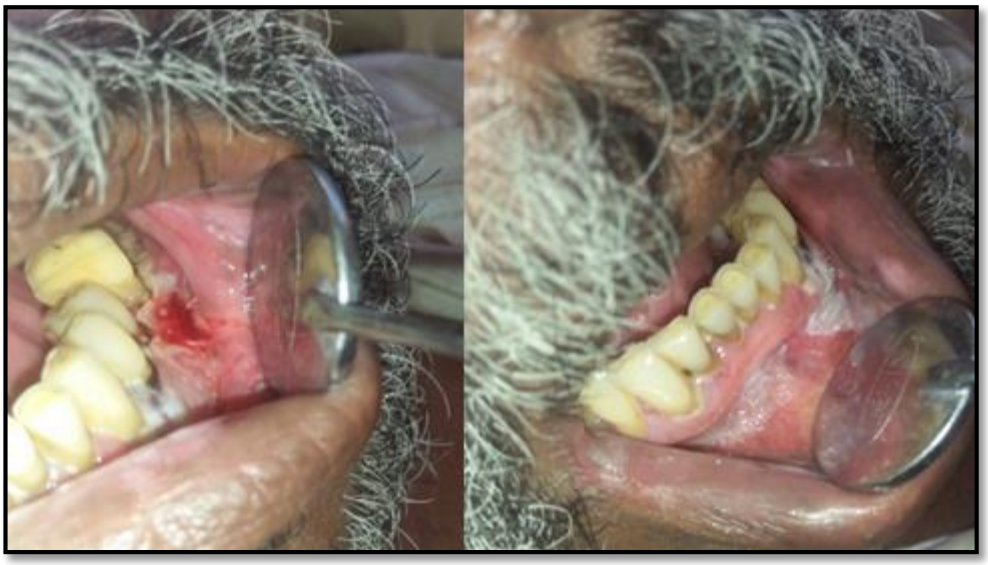


Photograph 5: Leukoplakia of right lateral border of tongue

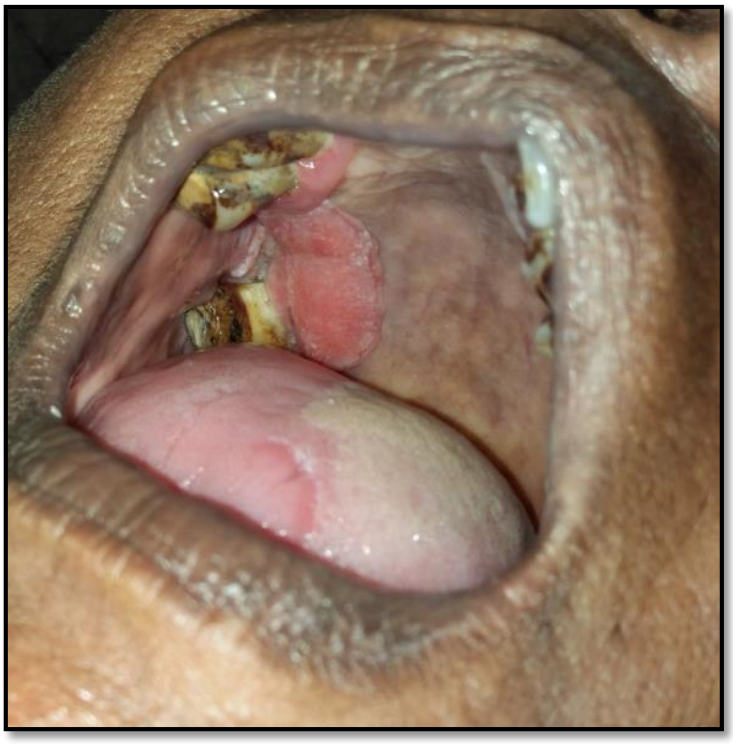


Photograph 6: Leukoplakia of left buccal mucosa

GROUP IV- TOBACCO ASSOCIATED ORAL SQUAMOUS CELL CARCINOMA

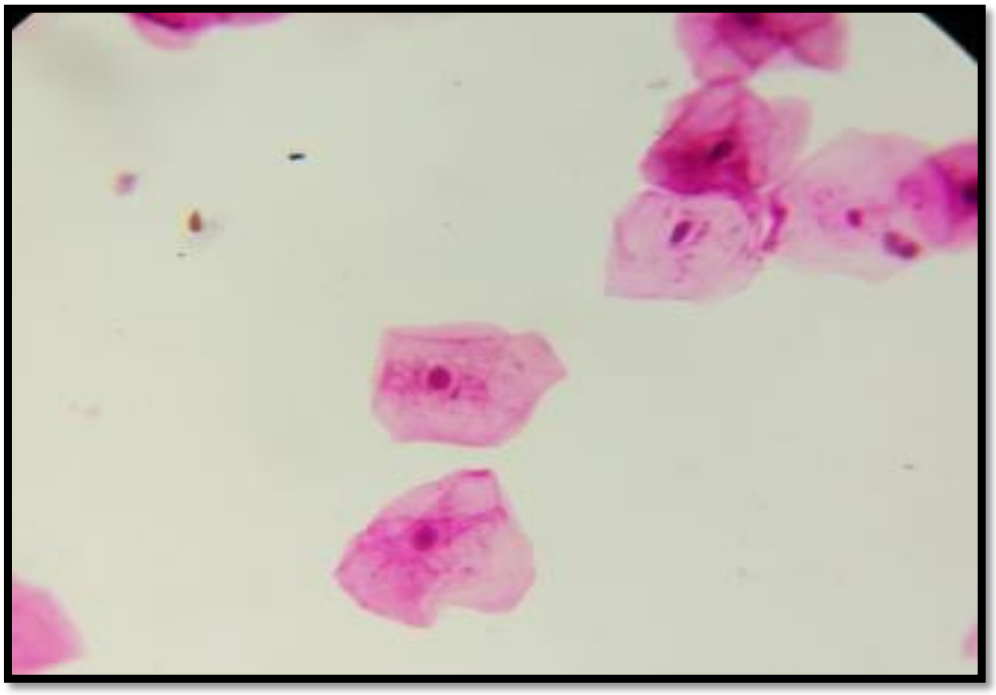


Photograph 7: Oral squamous cell carcinoma involving left buccal vestibule

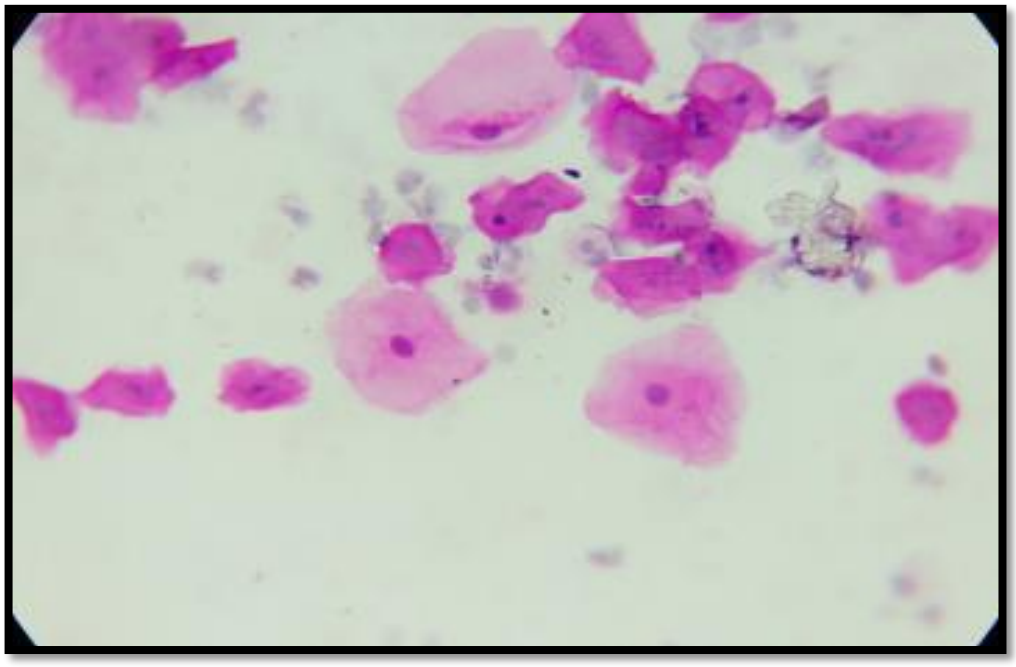


Photograph 8: Oral squamous cell carcinoma involving palate

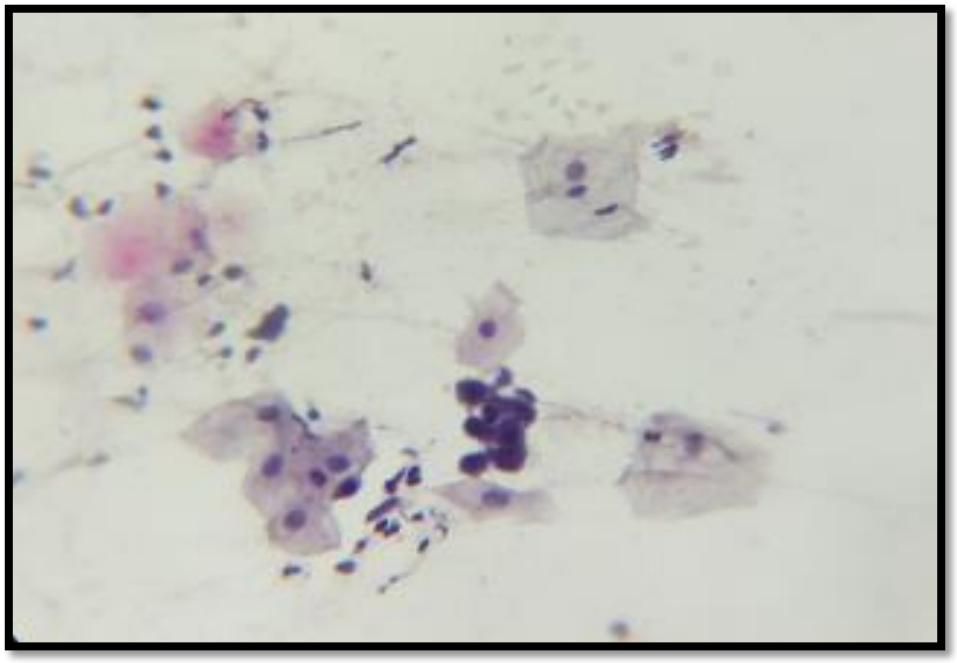
CYTOLOGY GRADES



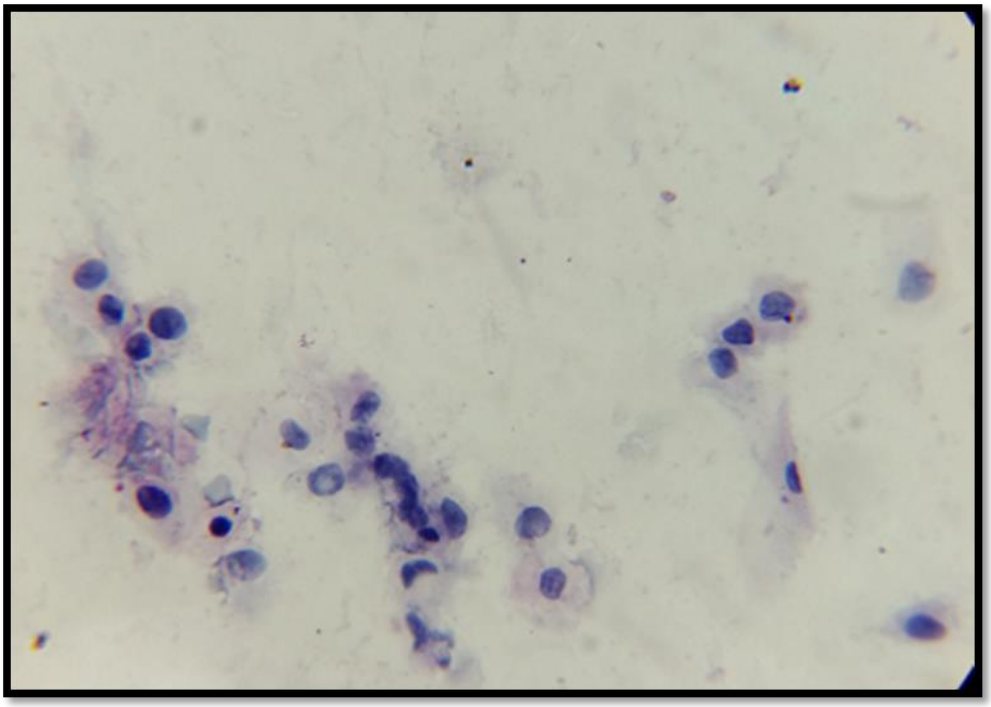
Photograph 9: Class I cytology smear



Photograph 10: Class II cytology smear

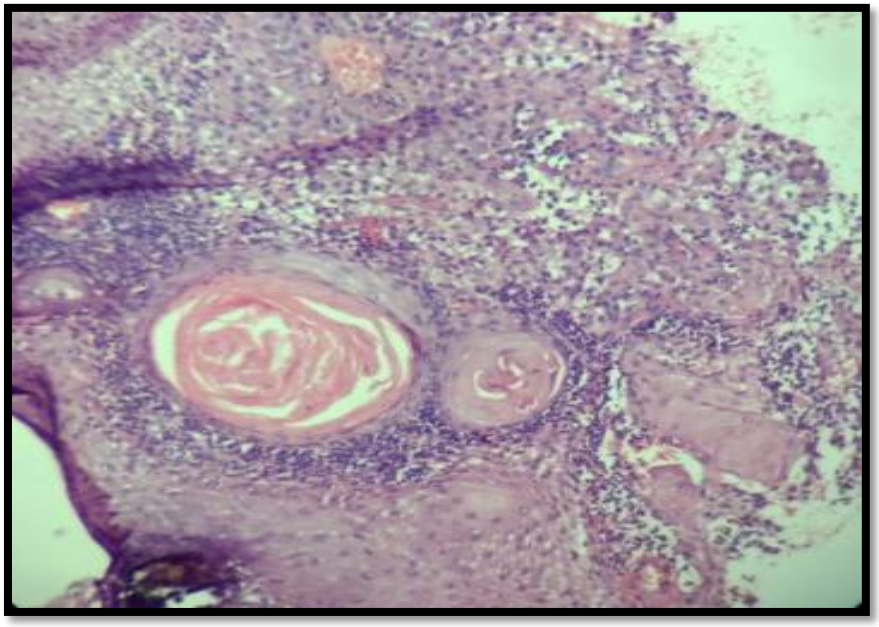


Photograph 11: Class III cytology smear

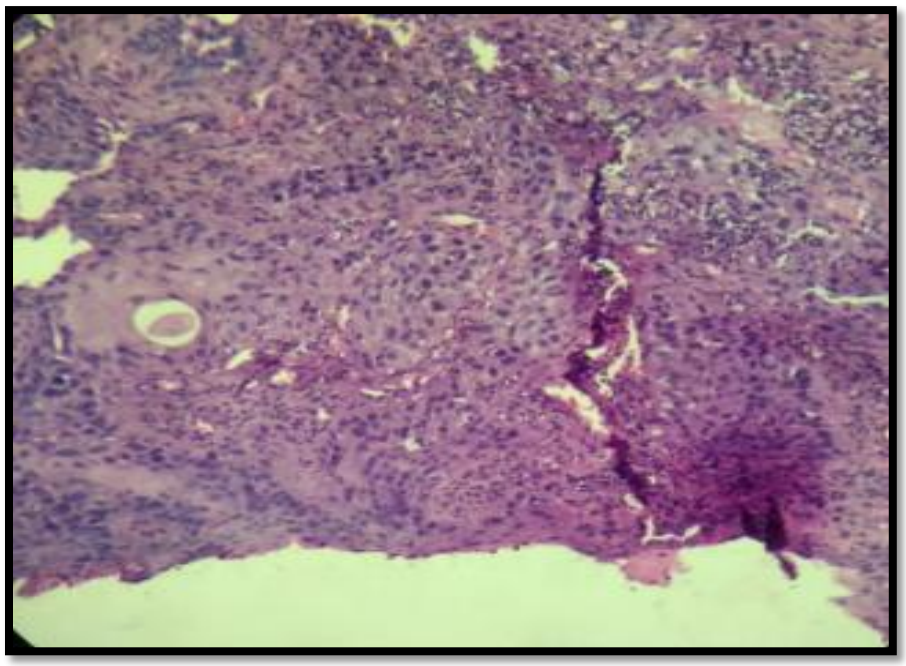


Photograph 12: Class IV cytology smear

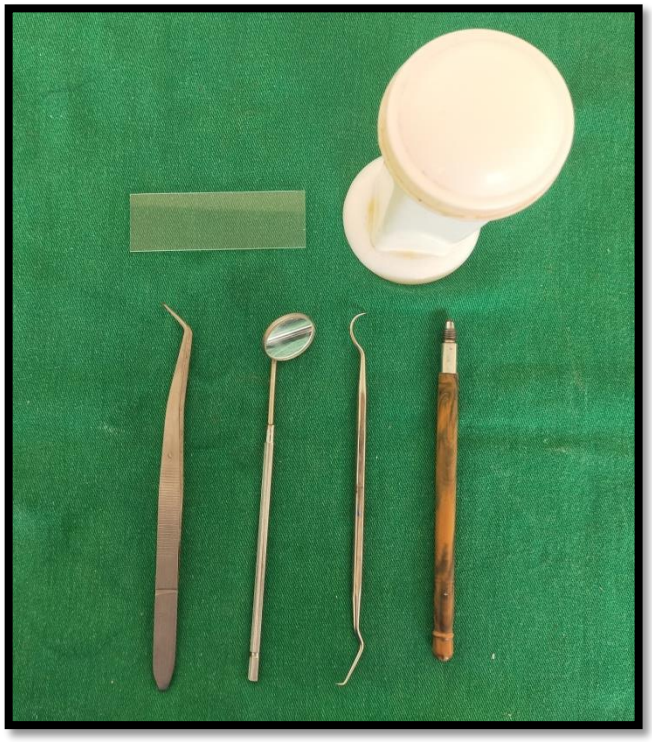
HISTOPATHOLOGY GRADES



Photograph 13: Well differentiated oral squamous cell carcinoma (40X)



Photograph 14: Moderately differentiated oral squamous cell carcinoma (40X)

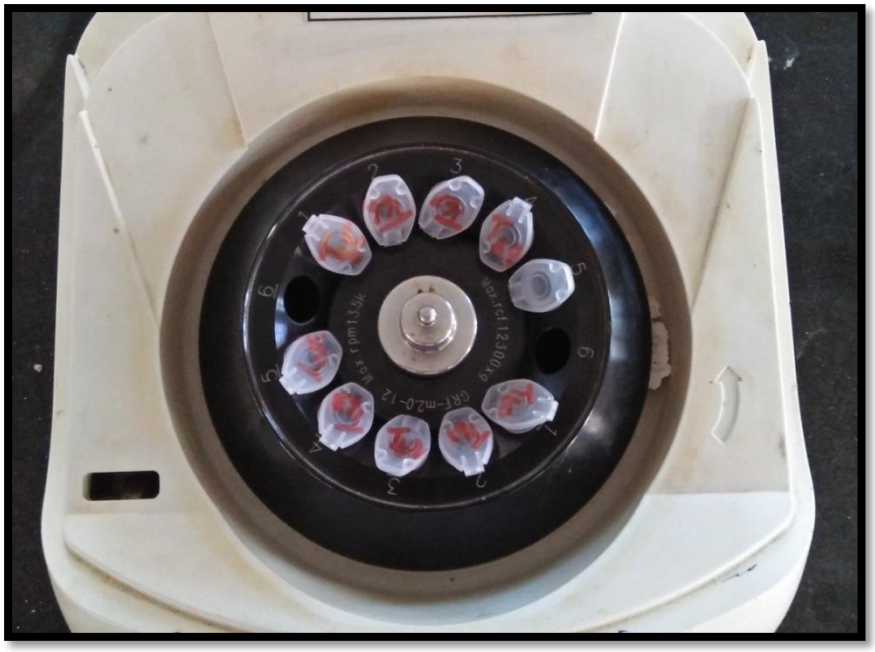


Photograph 15: Examination kit

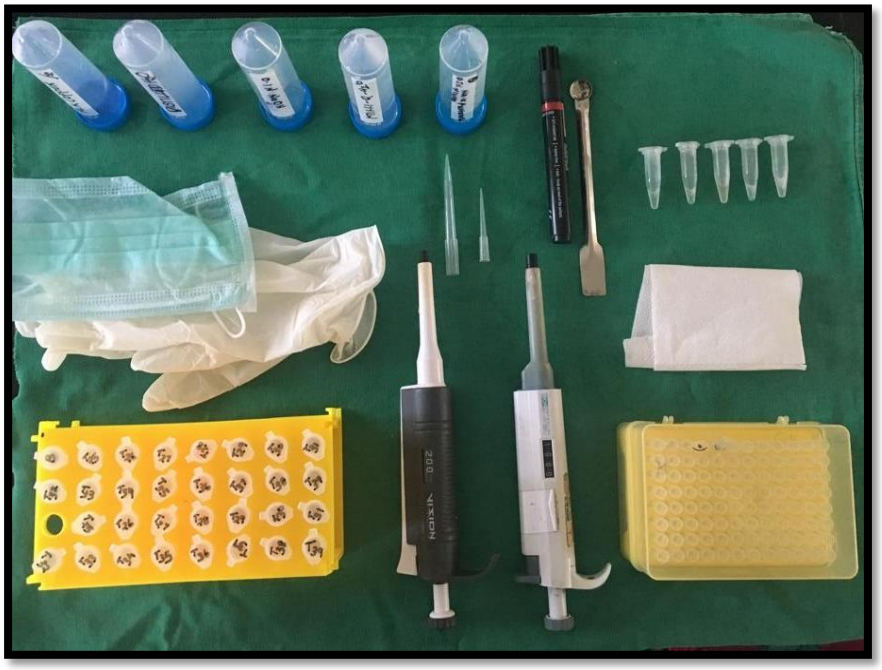
Photograph 16: Sterile saliva sample container

Photograph 17: Storage unit,-20 ºC freezer

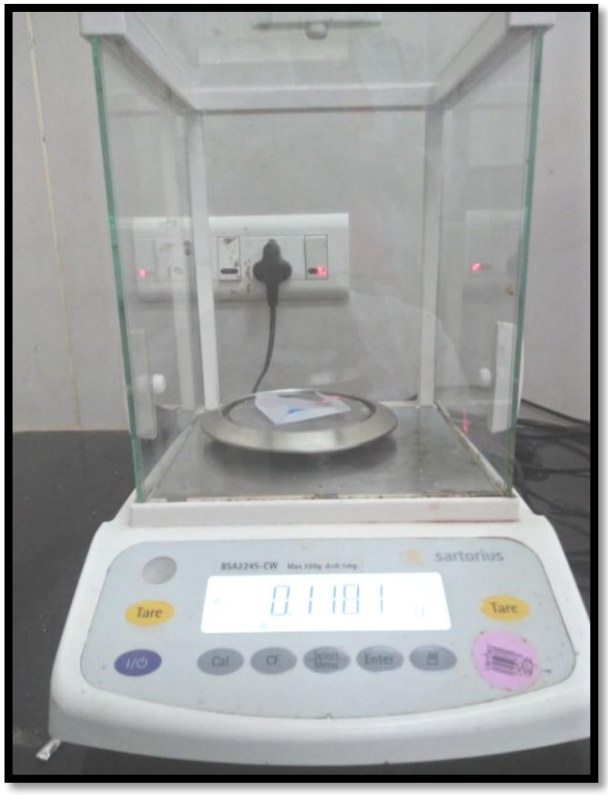
Photograph 18: Seggregated saliva samples



Photograph 19: Centrifuge loaded with Eppendorf tubes

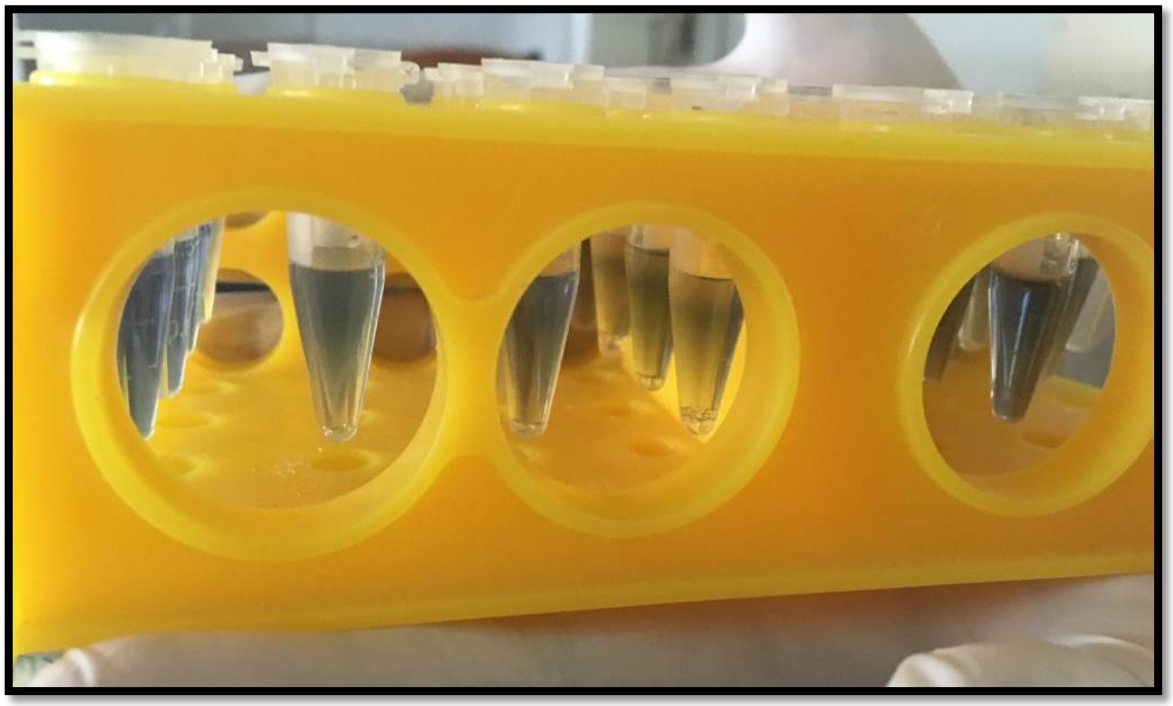


Photograph 20: Total protein estimation kit

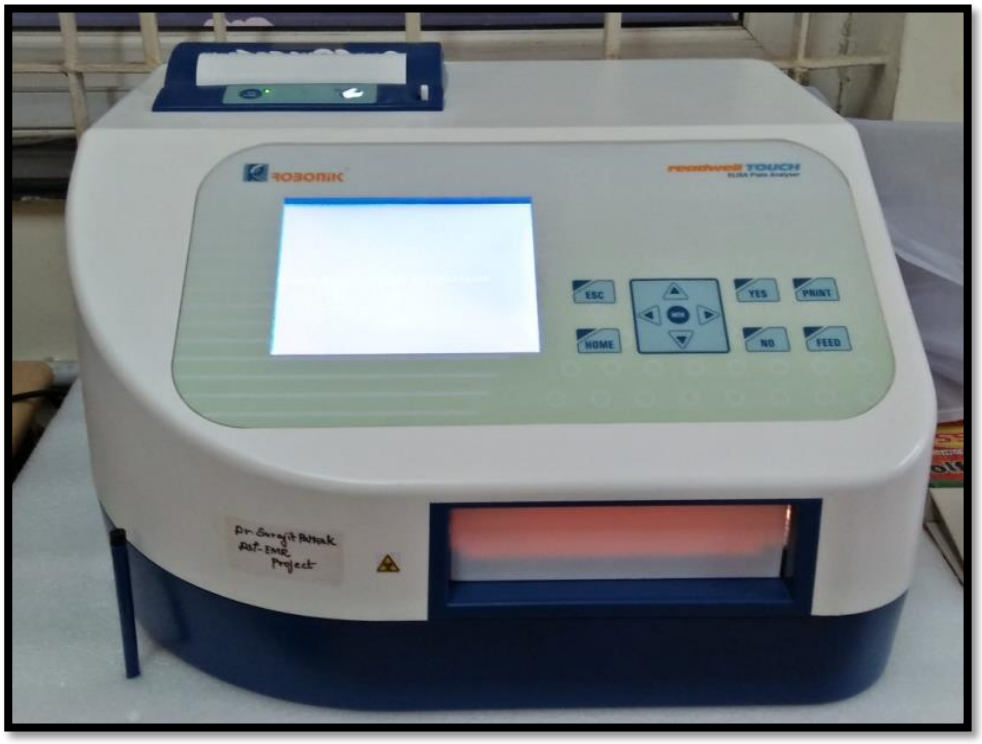


Photograph 21: Electronic weighing machine

Photograph 22: Reagents for total protein estimation



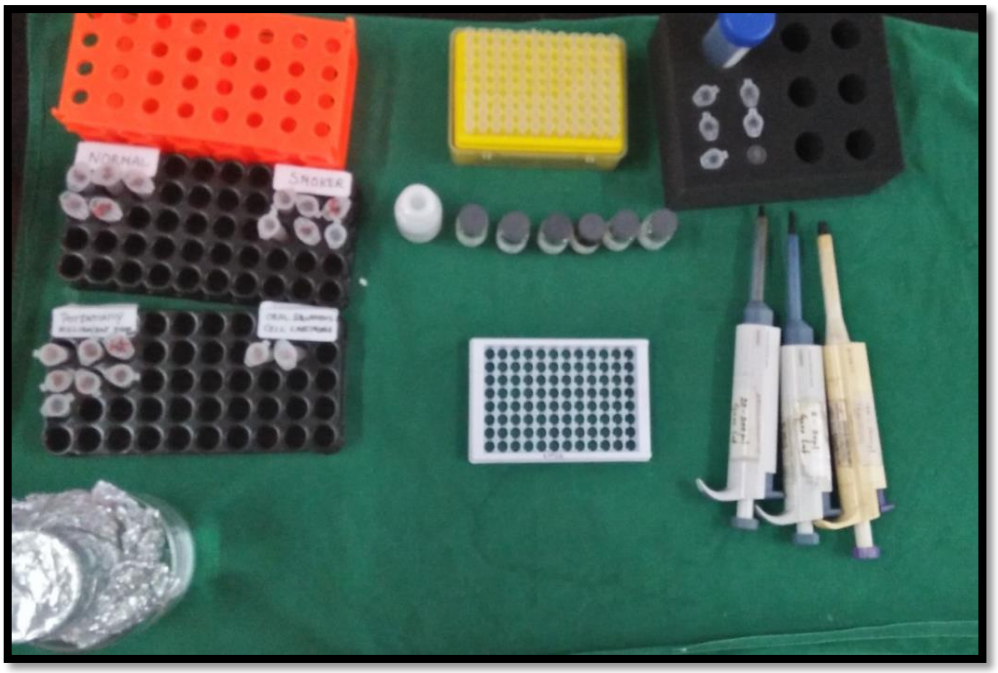
Photograph 23: Total protein analysis (colour change)



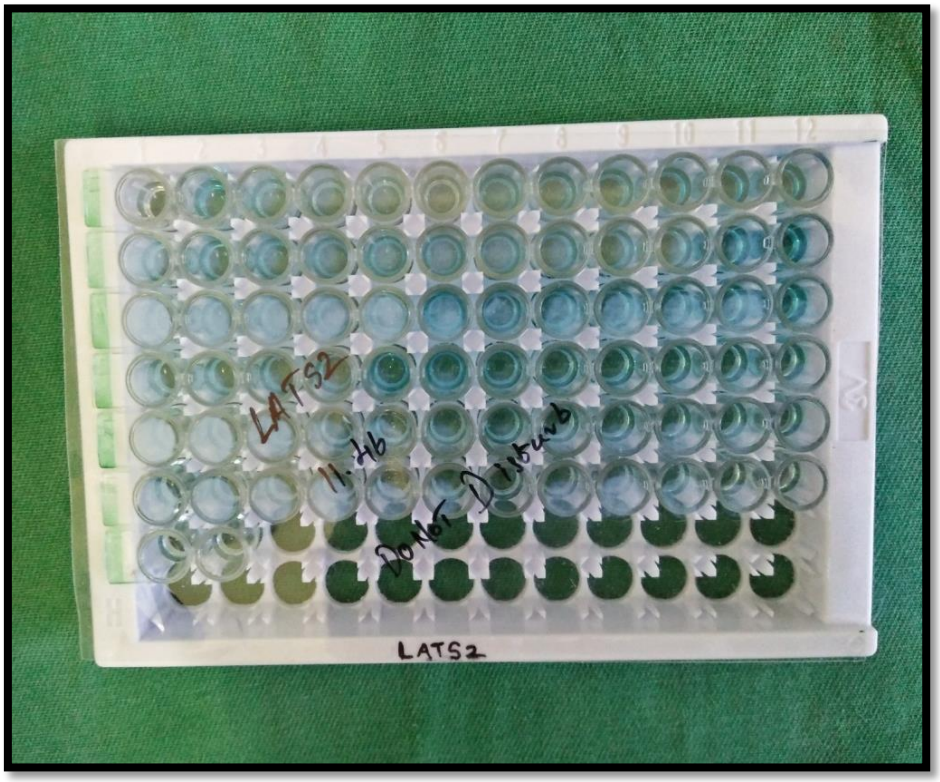
Photograph 24: Spectrophotometer

Photograph 25: LATS 1/2 ELISA kit reagents

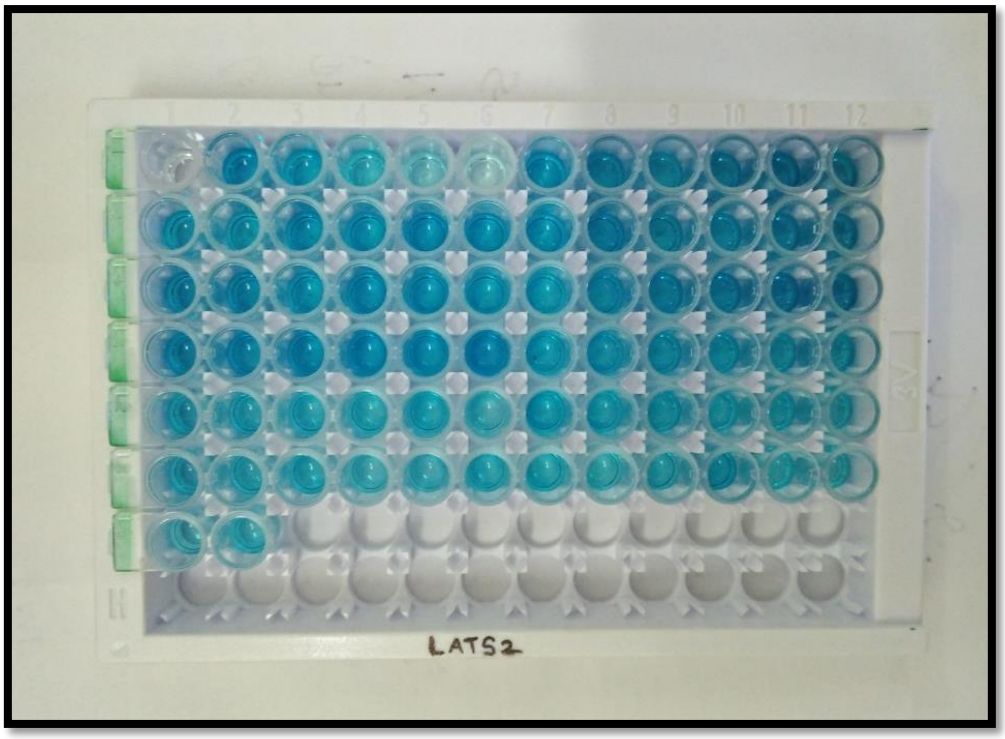
Photograph 26: Sterilized Milli-Q water



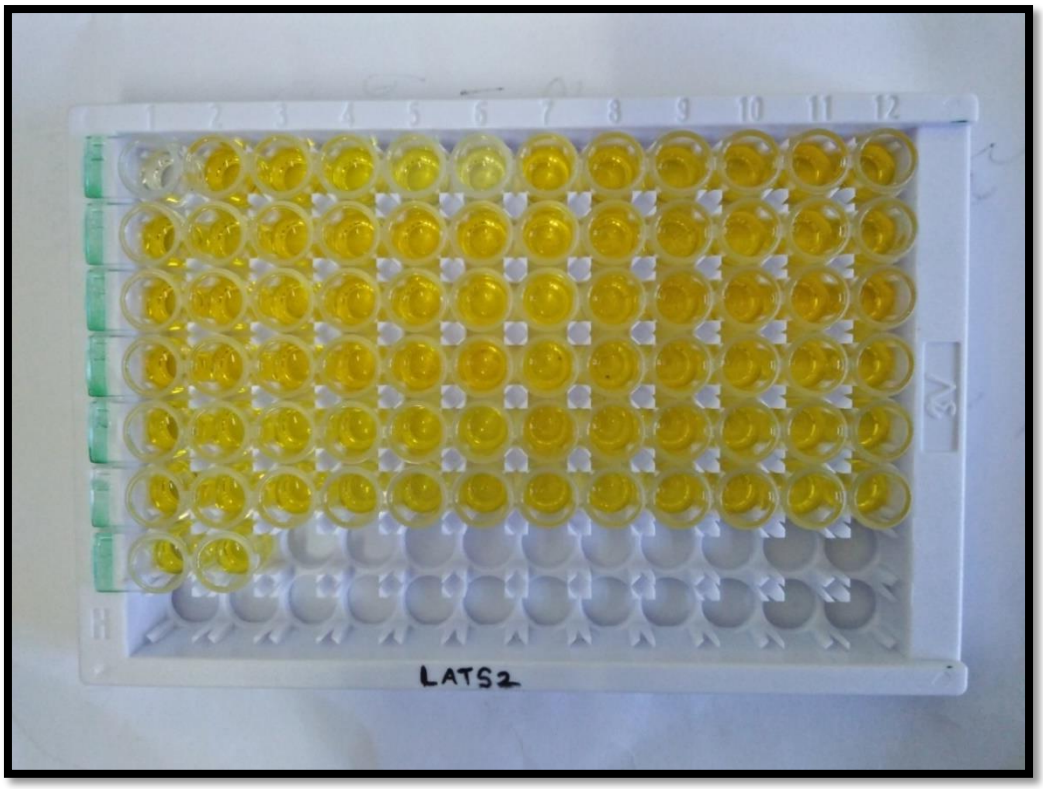
Photograph 27: Kit used for estimation of LATS using ELISA



Photograph 28: ELISA well coated with LATS antibody before incubation (light blue colour)



Photograph 29: ELISA well indicating the colour change after incubation (blue colour)



Photograph 30: ELISA wells after addition of stop solution confirming the presence of LATS (Yellow colour)

PARTICIPANT INFORMATION SHEET (PIS)

NAME OF THE INVESTIGATOR: Dr.Gowri.S

MOBILE NO: 9789886346

TITTLE: QUANTIFICATION OF LARGE TUMOR SUPPRESSOR KINASE 1 AND 2 IN TOBACCO ASSOCIATED POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA.

The purpose of the study is to quantify large tumor suppressor kinase 1 and 2 in saliva of patients with tobacco associated potentially malignant disorders and oral squamous cell carcinoma by ELISA. No risk will be involved to the patient as 3 ml of saliva sample will be collected in a non-invasive method. Details collected will be confidentially recorded and full freedom to participate or withdraw from the study at any point of time.

The detail of the study was explained to me in a language which I inderstood. I hereby give persmission for using my records and saliva sample for professional research and education purpose only.

SIGNATURE OF THE PATIENT:

பங்கேற்பு தகவல் தாள் (பிஐஎஸ்)

ஆய்வாளரின் பெயர்: டாக்டர். கௌரி.ஸ்

மொபைல் எண்: 9789886346

தலைப்பு: கூடியதாக உள்ள புற்றுத்திசு, புற்றுநோயில் லார்ஜ் டுயூமர் சப்ரஸார் கைனேஸ் 1 மற்றும் 2 மரபணு நிர்ணயித்தல்

ELISA ஆல் புகையிலை தொடர்புடைய வீரியம் மிக்க கோளாறுகள் மற்றும் வாய்வழி செதிள் உயிரணு புற்றுநோயால் பாதிக்கப்பட்ட நோயாளிகளின் உமிழ்நீரில் லார்ஜ் டுயூமர் சப்ரஸார் கைனேஸ் 1 மற்றும் 2 ஐ அளவிடுவதே ஆய்வின் நோக்கம். மூன்று மில்லி உமிழ்நீர் மாதிரி ஆக்கிரமிப்பு முறையில் சேகரிக்கப்படும் என்பதால் நோயாளிக்கு எந்த ஆபத்தும் ஏற்படாது. சேகரிக்கப்பட்ட விவரங்கள் ரகசியமாக பதிவு செய்யப்படும் மற்றும் எந்த நேரத்திலும் ஆய்வில் பங்கேற்க அல்லது விலகுவதற்கான முழு சுதந்திரமும் இருக்கும்.

ஆய்வின் விவரம் எனக்கு ஒரு மொழியில் விளக்கப்பட்டது. எனது பதிவுகள் மற்றும் உமிழ்நீர் மாதிரியை தொழில்முறை ஆராய்ச்சி மற்றும் கல்வி நோக்கத்திற்காக மட்டுமே பயன்படுத்துவதற்கு நான் இதன்மூலம் அனுமதிக்கிறேன்

நோயாளியின் அடையாளம்:

PARTICIPANT INFORMED CONSENT FORM (PICF)

IHEC Proposal S.No.: _____ Date:_____

Title of the project:
QUANTIFICATION OF LARGE TUMOR SUPPRESSOR KINASE 1 AND 2 IN TOBACCO ASSOCIATED POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA

Name of the Principal Investigator: DR.GOWRI S Mobile No.:9789886346

The contents of the information sheet dated _____ that was provided have been read carefully by me / explained in detail to me, in a language that I comprehend, and I have fully understood the contents. I confirm that I have had the opportunity to ask questions.

The nature and purpose of the study and its potential risks / benefits and expected duration of the study, and other relevant details of the study have been explained to me in detail. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal right being affected.

I understand that the information collected about me from my participation in this research and sections of any of my medical notes may be looked at by responsible individuals from CARE. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

Date:

_____ Place: (Signatures /Left Thumb Impression)

Name of the Participant:_____
Son / Daughter / Spouse of:_____
Complete Postal Address: _____

This is to certify that the above consent has been obtained in my presence.

Date:

Place:

_____
Signature of the principal Investigator

1. Witness – 1

_____
Signature

Name & Address

2. Witness – 2

_____
Signature

Name & Address

Note 1: Three copies should be made, for (a) Participant, (b) Researcher, (c) Institution
2: Submit the modified participants informend consent as per the study proposal
3: Investigators are advised to prepare the translation in simple understandable Tamil on their own

முறையான அனுமதி படிவம்

தேதி:

தலைப்பு: "கூடியதாக உள்ள புற்றுத்திசு , புற்றுநோயில் லத்ஸ் 1 மற்றும் லத்ஸ் 2 மரபணு நிர்ணயித்தல்"

முதன்மை ஆய்வாளரின் பெயர்: கௌரி.ஸ்

கைபேசி: 9789886346

இந்த தகவல் தாளின் உள்ளடக்கங்களை நான் கவனமாக படித்தேன். அது என்னுடைய மொழியில் எனக்கு விளக்கப்பட்டது நான் அவற்றை முழுமையாகப் புரிந்து கொண்டேன் எனக்கு ஏற்பட்ட சந்தேகங்களை தீர்த்துக்கொள்ள வாய்ப்பு அளிக்கப்பட்டது என்பதை உறுதி படுத்துகிறேன்.

இந்த ஆராய்ச்சியின் முடிவில் என்னுடைய பெயர் மற்றும் என்னை பற்றிய தகவல்கள் வெளியே வராது என்பதையும் நான் விரும்பும் பட்சத்தில் இவ்வராய்ச்சியிலிருந்து எப்பொழுது வேண்டுமானாலும் விலகி கொள்ளலாம் என்றும் அவ்வாறு விலகும் பொழுது அது எனக்கு அளிக்கப்படும் சிகிச்சையை ஒரு பொழுதும் பாதிக்காது என்றும் அறிந்துகொண்டேன்

இந்த ஆராய்ச்சியில் பங்கேற்பதற்கு முழுமனதாக நான் சம்மதிக்கின்றேன்.

நோயாளியின் கையப்பம் / இடது பெரு விரல்ரேகை:

ஆராய்ச்சியாளரின் கையப்பம்:

பங்கேற்பவரின் பெயர்: _____

பங்கேற்பவரின் முகவரி:

_____

_____

_____

சாட்சி(1)

பெயர்:
முகவரி:
கையப்பம்

சாட்சி(2)

பெயர்:
முகவரி:
கையப்பம்

NAME: **OP NO:**

AGE: **RMCT NO:**

SEX:

ADDRESS:

OCCUPATION:

CHIEF COMPLAINT:

HISTORY OF PRESENTING ILLNESS:

PAST MEDICAL HISTORY:

PAST DENTAL HISTORY:

FAMILY HISTORY:

GENERAL EXAMINATION:

VITALS:

PULSE RATE-

BLOOD PRESSURE-

RESPIRATORY RATE-

REVIEW OF SYSTEMS:

CNS:

RS:

CVS:

GIT:

UT:

LOCAL EXAMINATION:

EXTRA ORAL:

TMJ:

JAW MOVEMENTS:

MASTICATORY MUSCLES:

REGIONAL LYMPH NODES:

INTRA ORAL:

JAW MOVEMENT AND MOUTH OPENING:

TEEETH:

LIP:

GINGIVA:

ALVEOLAR MUCOSA:

BUCCAL MUCOSA:

PALATE:

TONGUE:

FLOOR OF THE MOUTH:

INSPECTION:

PALPATION:

SUMMARY:

DIAGNOSIS:

TEST SAMPLE NO	OP NO	AGE/ GENDER	GROUP	LESIONAL SITE	CLINICAL DIAGNOSIS	CYTOLOGY/HP GRADING	CYTOLOGY/HP NUMBER	LATS1 (ng/ml)	LATS2 (ng/ml)
T1	271203	25/F	Normal control	Right buccal mucosa	Apparently normal buccal mucosa	Class I	7006/18	2.699625	6.414822
T2	248297	23/M	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	7011/18	3.230858	7.340348
T3	270466	32/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	7014/18	3.636494	8.236759
T4	270655	23/F	Normal control	Right buccal mucosa	Apparently normal buccal mucosa	Class I	6997/18	0.940043	10.40984
T5	270075	22/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	6999/18	2.795874	8.865854
T6	268345	40/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	6858/18	3.055024	14.69352
T7	271101	25/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	7029/18	2.655356	7.810303
T8	270662	23/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	6994/18	1.72371	10.79531
T9	271068	23/F	Normal control	Left buccal mucosa	Apparently normal buccal mucosa	Class I	6992/18	3.021449	15.32986
T10	229353	23/F	Normal control	Right buccal mucosa	Apparently normal buccal mucosa	Class I	7051/18	3.289055	9.196038
T11	255415	32/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	7258/18	4.362462	8.010643
T12	255400	27/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	7342/18	3.629282	11.87379
T13	256548	28/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	8530/19	2.791149	6.391675
T14	194778	56/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class I	7267/18	2.811294	3.681007
T15	300324	26/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	8459/19	3.477821	7.99326
T16	324684	29/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	8238/19	4.047851	5.875931
T17	325466	32/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class III	7352/18	3.222153	9.426108
T18	325353	63/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class I	8253/19	4.054069	6.819182
T19	325917	41/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class I	7657/18	2.523045	7.759533
T20	289294	43/M	Smokers without lesion	Buccal mucosa	Smoker with pigmentation	Class II	8246/19	1.908746	6.411472
T21	267131	51/M	Potentially malignant disorder	Right and left buccal mucosa	Leukoplakia in relation to right and left buccal muca	Class II	8373/19	4.919311	1.534059
T22	321183	59/M	Potentially malignant disorder	Left buccal mucosa	Leukoplakia in relation to left buccal mucosas	Class I	9444/19	4.439063	6.217308
T23	309818	56/M	Potentially malignant disorder	Left buccal mucosa	Leukoplakia in relation to left buccal mucosa	Class IV	8997/19	3.661862	6.007483
T24	310356	32/M	Potentially malignant disorder	Right and left alveolar region	Leukoplakia in relation to right and left alveolar region	Class I	8960/19	5.489838	15.01698
T25	217005	53/F	Potentially malignant disorder	Right lateral border of tongue	Leukoplakia in relation to right lateral border of tongue	Class I	6246/18	4.959352	6.056815
T26	306368	59/M	Potentially malignant disorder	Right buccal mucosa and vestibular region	Leukoplakia in relation to right buccal mucosa and vestibule	Class II	8758/19	4.218711	27.90364
T27	274841	39/M	Potentially malignant disorder	Left buccal mucosa	Leukoplakia in relation to left buccal mucsa	Class II	8054/19	4.77357	1.175374
T28	260888	38/M	Potentially malignant disorder	Right and the left vestibular region	Leukoplakia in relation to right and left vestibular region	Class II	9375/19	4.574855	8.751767
T29	321437	32/M	Potentially malignant disorder	Right buccal mucosa	Leukoplakia in relation to right buccal mucsa	Class I	9460/19	4.155291	26.51884
T30	302433	62/M	Potentially malignant disorder	Right buccal mucosa	Leukoplakia in relation to right buccal mucsa	Class III	8821/19	4.250048	9.056118
T31	253752	48/M	Oral squamous cell carcinoma	Right floor of the mouth	Soft tissue malignant neoplasm in right floor of oral cavity.	Class III/ Moderately differentiated	2046/18	3.611127	3.271901
T32	301851	42/M	Oral squamous cell carcinoma	Right buccal mucosa	Traumatic ulcer in right buccal mucosa	Class III/ Moderately differentiated	2696/19	5.714418	7.438111
T33	201803090022	49/M	Oral squamous cell carcinoma	Right lateral border of tongue	Malignant ulcer in right lateral border of tongue.	Class IV/ Well differentiated	2027/18	3.407935	4.398682
T34	201803130030	30/M	Oral squamous cell carcinoma	Right buccal mucosa	Soft tissue malignant neoplasm in right buccal mucosa.	Class IV/ Moderately differentiated	2000/18	6.366521	6.551566
T35	255430	52/M	Oral squamous cell carcinoma	Left lower vestibule	Malignant soft tissue neoplasm in left lower vestibule.	Class II/ Well differentiated	2069/18	4.691995	1.445092
T36	234240	35/F	Oral squamous cell carcinoma	Left alveolus	Malignant ulcero proliferative growth in left mandibular alveolus.	Class IV/ Well differentiated	5040/17	6.207847	1.545692
T37	234246	45/F	Oral squamous cell carcinoma	Right lateral border of tongue	Traumatic ulcer	Class II/ Well differentiated	5039/17	3.981696	3.582742
T38	268343	40/F	Oral squamous cell carcinoma	Right buccal mucosa	Verrucous carcinoma of right buccal complex of mandible.	Class IV/ Well differentiated	2242/18	6.214562	0.788612
T39	268484	43/M	Oral squamous cell carcinoma	Left buccal mucosa	Verrucous carcinoma in left buccal mucosa.	Class III/ Well differentiated	2224/18	5.23044	0.973257
T40	233747	50/F	Oral squamous cell carcinoma	Right side of palate	Malignant ulcer in relation to 17	Class III/ Moderately differentiated	5016/17	5.097383	0.85336