IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY

DISSERTATION

SUBMITTED TOTHE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI

In partial fulfilment of
the requirements for the degree of

M.D. (PATHOLOGY)

BRANCH – III

DEPARTMENT OF PATHOLOGY

TIRUNELVELI MEDICAL COLLEGE HOSPITAL

TIRUNELVELI - 627011

APRIL-2016

CERTIFICATE

This is to certify that this Dissertation entitled "**IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY**" is the bonafide original work of **DR.SANKAR GANESH.T,** during the period of her Post graduate study from September 2013to September 2015, under my guidance and supervision, in the Department of Pathology Tirunelveli Medical College& Hospital, Tirunelveli, in partial fulfilment of the requirement for M.D., (Branch III)in Pathology examination of the TamilnaduDr.M.G.R Medical University to be held in April 2016.

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CERTIFICATE

I hereby certify that this dissertation entitled**"IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY"** is a record of work done by **DR.SANKAR GANESH.T,** in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during her postgraduate degree course period from 2013-2015.This work has not formed the basis for previous award of any degree.

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10

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PROTOCOL TITLE: Immunohistochemical analysis of P53 expression in oral precancerous and cancerous lesions and its relation with poor personal history

NAME OF PRINCIPAL INVESTIGATOR: Dr. Sankar Ganesh
DESIGNATION OF PRINCIPAL INVESTIGATOR: Resident in Pathology
DEPARTMENT & INSTITUTION: Department of Pathology, Tirunelveli Medical College

Dear Dr. Sankar Ganesh, *The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 28.12.13.*

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1.	TIREC Application Form	☑
2.	Study Protocol	☑
3.	Department Research Committee Approval	☑
4.	Patient Information Document and Consent Form in English and Vernacular Language	☑
5.	Investigator's Brochure	☐
6.	Proposed Methods for Patient Accrual Proposed	☑
7.	Curriculum Vitae of the Principal Investigator	☑
8.	Insurance /Compensation Policy	☑
9.	Investigator's Agreement with Sponsor	☐
10.	Investigator's Undertaking	☑
11.	DCGI/DGFT approval	☐
12.	Clinical Trial Agreement (CTA)	☐
13.	Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA)	☑
14.	Clinical Trials Registry-India (CTRI) Registration	☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
6. At the time of PI's retirement/leaving the institute, the study responsibility should be transferred to a person cleared by HOD
7. The PI should report to TIREC within 7 days of the occurrence of the SAE. If the SAE is Death, the Bioethics Cell should receive the SAE reporting form within 24 hours of the occurrence.
8. In the events of any protocol amendments, TIREC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. The PI must comment how proposed amendment will affect the ongoing trial. Alteration in the budgetary status, staff requirement should be clearly indicated and the revised budget form should be submitted.
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented.
   d. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IEC, only then can they be implemented.
   e. Approval for amendment changes must be obtained prior to implementation of changes.
   f. The amendment is unlikely to be approved by the IEC unless all the above information is provided.
   g. Any deviation/violation/waiver in the protocol must be informed

STANDS APPROVED UNDER SEAL

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DECLARATION

I solemnly declare that this dissertation titled**"IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY"** submitted by me for the degree of M.D, is the record work carried out by me during the period of 2013-2015 under the guidance of **PROF. DR.S.VALLI MANALAN,** Professor of Pathology, Department of Pathology, Tirunelveli Medical College, Tirunelveli. The dissertation is submitted to The TamilnaduDr. M.G.R. Medical University, Chennai, towards the partial fulfillment ofrequirements for the award of M.D. Degree (Branch III) Pathology examination to be held in April 2016.

Place: Tirunelveli

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ABBREVIATIONS	
APTS	Amino Propyl Triethoxy Saline
ASNA	Areca Nut Specific Nitrosamines
BCIP	BromoChloroTetrazolium
BSA	Bovine Serum Albumin
CDK	Cyclin Dependant Kinases
CIS	Carcinoma In Situ
CN	Cranial Nerve
CT	Computed Tomography
DAB	DiaminoBenzidine
DAPI	Diamino Phenyl Indole
DNA	DeoxyRibo Nucleic Acid
DPX	Distyrene Plasticizer And Xylene
EBER	Epstein Barr Virus Encoded Ribo Nucleic Acid
EBV	Epstein Barr Virus
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor Threonine Kinases
ENT	Ear Nose And Throat
ERK	Extracellular Signal Regulated Kinases
ERK	Epidermal Growth Factor Receptor Threonine Kinases
FNAC	Fine Needle Aspiration Cytology
GADD	Growth Arrest And Dna Damage Protein
H&E	Hematoxylin And Eosin
HCL	Hydro Chloric Acid
HIER	Heat Induced Epitope Retrieval
HPV	Human Papilloma Virus
HRP	Horse Radish Peroxidase
IHC	Immunohistochemistry
LFS	Li-Fraumini Syndrome
LOH	Loss Of Heterozygosity
MAP-2	Microtubule Associated Protein Kinase - 2
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MDM	Mouse Double Minute
MRI	Magnetic Resonance Imaging
NBT	Nitro Blue Tetrazolium
NMU	Nitroso Methyl Urea
OSCC	Oral Squamous Cell Carcinoma
PAS	Periodic Acid Schiff
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction

PIER	Proteolytic Enzyme Induced Epitope Retrieval
PKC	Protein Kinase C
RNA	Ribo-Nucleic Acid
ROS	Reactive Oxygen Species
RSK	Ribosomal S6 Protein Kinase
RTK	Receptor Tyrosine Kinases
SCC	Squamous Cell Carcinoma
SV	Simian Vacuolating Virus
TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
TNM	Tumor Node And Metastasis
TSNA	Tobacco Specific Nitrosamines
WHO	World Health Organization

IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY

ABSTRACT

Oncoprotein p53 actively participates in oral carcinogenesis, in its initiation and/or promotion of the process. The expression of mutant p53 has been shown to be present in many human solid tumours of squamous origin including oral squamous cell carcinomas. The wild-type p53 acts as a tumour suppressor recessive gene, the inactivation of which could lead to malignant transformation. The wild-type p53 protein appears to block the cell cycle at the GUS boundary of the cell cycle and to produce GI arrest which provides sufficient time for DNA repair. On the other hand, its mutant variety forms complexes with the wild-type, inactivates the wild-type, and thus prevents the wild-type from carrying out its normal function. This may, in turn, lead to abnormalities in the cell cycle repair system andeventually to malignant transformation. Mutations in p53 gene may provide the cells with a growth advantage over the normal cells leading to malignant transformation. This study investigated the expression of oncoprotein p53 in oral cavity lesions s (60 samples) from tertiary care hospital Tirunelveli medical college – Tirunelveli, using monoclonal antibodies with the polymer-HRP (Horseradish peroxidase) indirect immunohistochemical staining methods. 46/60 Cases were positive for anti-p53 antibody staining and most of the positive specimens showed diffuse pattern of staining, where the cells were seen as small islands. Statistical analysis shows that there is a significant correlation between the expression of p53, cigarette/beedi smoking and the tobacco chewing habits.As the mutant oncoprotein p53 seems to play a role in malignant transformation, more cells are likely to show a strong nuclear staining reaction when compared to normal or hyperplastic oral epithelia. The significance of p53 oncoprotein in head and neck tumourigenesis is yet to be understood. Further studies such as Direct Nucleotide Sequencing, Polymerase Chain Reaction (PCR) and Single Strand Conformation Polymorphism (SSCP), addressing the possible involvement of this oncoprotein are warranted to get an insight into the molecular details of the mechanism.

KEY WORDS :

Oral squamous cell carcinoma, p53 oncoprotein, smoking, tobacco chewing.

INTRODUCTION

Cancer is the second most common cause of mortality in the world today. Totally 8 lakh new cases of cancer are reported every year in India of which 48% of cases among men and 20% among women are tobacco related cancer which is preventable. Incidence rate of cancers of oral cavity is 7.2 for men and 4.3 for women in India (1). Smoking tobacco, alcohol consumption, betel quid chewing with or without tobacco are the implicated as the principal causes for cancers of the oral cavity (2).

Numerous carcinogens are present within the smoke and smokeless form of tobacco products. These carcinogens combine with the human DNA and forms DNA adducts. During replication these DNA adducts leads to mutations and derange the cellular growth control processes (3). p53 gene is one of the important gene involved in cellular growth and is one of the most common gene mutated in malignant lesions (4). Various studies have reported p53 gene over expression in premalignant and malignant lesions of the oral cavity (5). These p53 gene overexpression can be identified by immunohistochemistry by immunostaining the paraffin embedded tissue blocks from the lesions of the oral cavity. This study analyses the p53 expression in lesions of the oral cavity and its relationship with tobacco smoking, chewing betel quid with or without tobacco.

AIMS AND OBJECTIVESOF THE STUDY

Most of the studies have suggested that there is a six-fold increase in the expression of p53 oncoprotein in Oral Squamous cell carcinomas (OSCCs). On the contrary, one of the studies conducted by Ranasinghe et al. (1993) stated that the expression of p53 oncoprotein in oral squamous cell carcinomas is very low (6). The objective of this study is to determine the extent and pattern of expression of the oncoprotein p53 in lesions of oral cavity samples from Tirunelveli, Southern India, where the population is exposed to the same etiologic factors in the same geographical location.

1. To collect data from cases with lesions of oral cavity sent to The Department of Pathology, Tirunelveli Medical College-Tirunelveli for histopathological evaluation during the study period.
2. To categorize them into non-malignant, premalignant and malignant lesions.
3. To collect history of tobacco smoking, betel nut chewing in patients with lesions of the oral cavity.
4. To assess incidence and distribution of malignancy in oral cavity based on age, sex, site and habitual history.
5. To do p53 immunostaining on these cases and assess the expression of p53expressionon these lesions.
6. To assess the correlation between p53 expression and history of tobacco smoking and betel quid chewing.

REVIEW OF LITERATURE

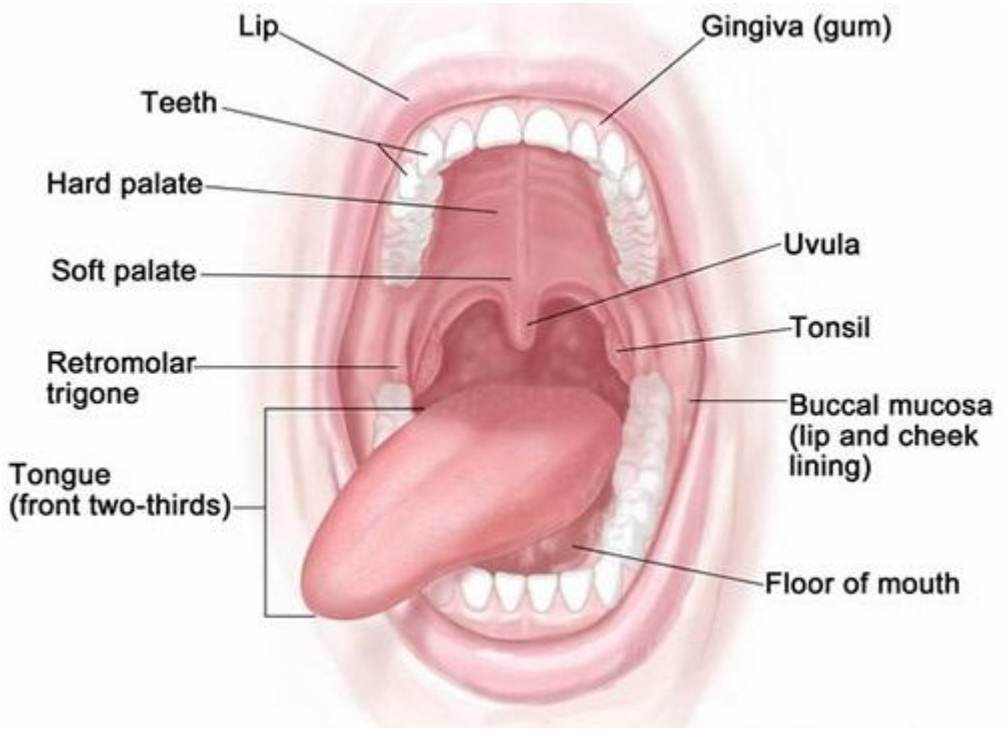
REVIEW OF LITERATURE

ANATOMY AND HISTOLOGY

ORAL CAVITY

The oral cavity refers to the internal part of the mouth and can be divided into the oralvestibule andthe oral cavity proper. The oral vestibule is the space between the inner lips, cheeks, and front surface of the teeth. The oral cavity proper is the space between the upper and lower dental arches, extending from the inner surface of the teeth to the oropharynx.

Figure - 1.



The structures inside the oral cavity include the lips, cheeks, tongue, teeth, gingiva, palates (hard and soft), salivary glands, and tonsils(fig – 1). The structures in the oral cavity are lined by an oral mucosa, which includes an overlying epithelium and underlying connective tissue. The oral mucosa can be divided into three types based on differences in the epithelial covering, organization of the connective tissue, and associated functions: lining, masticatory, and specialized mucosa.

LINING MUCOSA is covered by nonkeratinized stratified squamous epithelium with two distinct layers: the stratum basale and stratum spinosum. The epithelium of the lining mucosa is similar to the epidermis of the skin, except that it has neither a stratum corneum nor a stratum lucidum, and the stratum granulosum is often absent. The nonkeratinized stratified squamous epithelium is moistened by saliva secreted from salivary glands. The connective tissue adjacent to the lining mucosa can be divided into the lamina propria and the submucosa. The lamina propria is a thin layer of loose connective tissue containing many elastic fibres and relatively few collagen fibres. This layer is equivalent to the dermis of the skin and is located beneath the epithelium. The submucosa is a thick layer of connective tissue, which contains minor salivary glands and is attached to the underlying muscle. The lining mucosa covers the inner oral surfaces of the lips, cheeks, soft palate, the inferior surface of the tongue, and the floor of the mouth. This type of mucosa is less exposed to abrasion than the masticatory mucosa. The lining mucosa provides a barrier against the invasion of pathogens and toxic chemicals, contains receptors for sensations, and serves immunological functions. The lining mucosa also provides lubrication and buffering

by minor glands in the submucosal layer. Examples of the lining mucosa include the lip and cheek.

MASTICATORY MUCOSA is covered by keratinized stratified squamous epithelium, which is exposed to significant abrasion due to high compression and friction during chewing. The epithelium of the masticatory mucosa is composed of the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. It has a thick lamina propria that contains a dense network of collagen fibres and a few elastic fibres. This layer has no submucosa and is directly and firmly attached to the underlying bone. Masticatory mucosa can be found covering the oral surfaces of the gingiva and the hard palate. Injection into this area is difficult and painful because of its sensitive periosteum, high collagen density, and firm attachment to the bone.

SPECIALIZED MUCOSA covers the anterior two thirds of the tongue and consists of keratinized and nonkeratinized squamous epithelium and numerous papillae. These papillae can be classified into four types: filiform, fungiform, circumvallate, and foliate papillae. Most of these papillae have taste buds. The filiform papillae are the only papillae without taste buds; their main function is to aid in mixing food during chewing.

The lamina propria (connective tissue) of the specialized mucosa is attached to the underlying skeletal muscle. These muscles produce voluntary movement of the tongue and are innervated by the hypoglossal nerve (cranial nerve [CN] XII). Lining mucosa covers the inferior surface of the tongue. The mucosa of the tongue is divided into two parts by a V-shaped groove called the sulcus terminalis. The anterior two thirds of the

tongue is referred to as the body of the tongue. Its mucosa is innervated by the facial nerve (CN VII) and the trigeminal nerve (CN V). The posterior third of the tongue is the base of the tongue. Its taste buds and mucosa are innervated by the glossopharyngeal nerve (CN IX). The posterior third of the tongue contains the lingual tonsils

1. Filiform papillae are the smallest and most numerous of the four types of papillae. They cover almost the entire superior surface of the anterior two thirds of the tongue and are packed in rows that parallel the sulcus terminalis. Each of the papillae appears cone shaped with some branching processes. Connective tissue forms the central core of each papilla. Filiform papillae have no taste buds and extend from the nonkeratinized stratified squamous epithelium. The surface of the papilla is keratinized and is exposed to a great deal of abrasion.

2. Fungiform papillae are less numerous than the filiform papillae. They are mushroom shaped and are scattered among the filiform papillae. Fungiform papillae are located at the tip and on the two lateral edges of the tongue. They are more numerous near the tip of the tongue. Taste buds are found on the apical surfaces of fungiform papillae.

3. Circumvallate papillae are large and round with a flat topped cylindrical structure. There are about 10 to 14 papillae arranged in a row along the sulcus terminalis. Each papilla is surrounded by a deep groove (moat), which forms a valley around the papilla. Taste buds are found in the lateral walls of each papilla.

4. Foliate papillae are leaf like folds with flat tops and have deep clefts between the papillae. They are located on the posterior lateral surface of the tongue. They are more prominent in some animals (such as rabbits) than in humans. Foliate papillae contain taste buds in the lateral walls of the papillae.

UPPER RESPIRATORY AIRWAY

The upper respiratory airway functions as a part of the conducting portion; it consists of the nasal cavity, nasopharynx, oropharynx, and larynx. In general, the conducting airway is composed of bone, cartilage, and fibrous tissue and is lined with stratified squamous and ciliated pseudostratified columnar epithelia moistened with mucus and other glandular secretions. Cilia on the surface of the pseudostratified columnar epithelia sweep particles out of the respiratory airway.

THE NASAL CAVITY is the first portion of the upper respiratory airway. It can be divided into three regions based on the types of epithelial coverings.

1. The nasal vestibule is the most anterior part of the nasal cavity and is covered by a keratinized stratified squamous epithelium and vibrissae (stiff hairs); it is continuous with a mucosa of nonkeratinized stratified squamous epithelium.
2. The nasal mucosa region is covered by pseudostratified ciliated epithelium (respiratory epithelium), which contains ciliated columnar cells, goblet cells, basal cells, and, occasionally, neuroendocrine cells. The goblet cells manufacture mucus, which traps particles of dust and bacteria and moves them out of the nasal fossa, sinuses, and the nasopharynx, with the help of the ciliary action of the epithelium. Nasal mucosa filters, warms, and moistens the inhaled

air. Mucus serves as a protective mechanism for preventing pathogens and irritants from entering the respiratory airway. There is a special vascular arrangement in the lamina propria of the nasal conchae called swell bodies (venous plexuses), which alternately fill with blood from the small arteries directly into the venous plexuses on each side of the nasal cavity to help reduce airflow and increase air contact with nasal mucosa.

3. The olfactory mucosa region is located in the roof of the nasal cavity and is covered by pseudostratified columnar epithelium, which is composed of ciliated olfactory cells (olfactory receptor neurons), nonciliated columnar cells, and basal cells. It functions as a site for odorant chemoreception.

THE NASOPHARYNX AND OROPHARYNX conducts air from the nasal cavity and oral cavity to the larynx. The oropharynx is lined by stratified squamous epithelium, and the nasopharynx is lined by respiratory (pseudostratified columnar) epithelium. The nasopharynx contains seromucous glands in the lamina propria. The pharyngeal tonsil, an unencapsulated patch of lymphoid tissue, is located in the posterior aspect of the nasopharynx. The palatine tonsils are located at the junction of the oral cavity and the oral pharynx, between the palatoglossal and the palatopharyngeal folds, which indicate the posterior boundary of the oral cavity. Tonsils, rich in lymphoid tissue, are the first line of defense against many airborne pathogens and irritants.

THE LARYNX conducts air from the pharynx to the trachea. It is supported by a set of cartilages of complex shape and covered by a ciliated, pseudostratified respiratory epithelium. This mucosa continues from that of the pharynx and extends to the

trachea. The larynx contains several structures, including the epiglottis, vocal cords, and nine pieces of cartilage located in its wall. The epiglottis is a thin leaf like plate structure; its central cord contains a large piece of elastic cartilage. This cartilage is attached to the root of the tongue and projects obliquely upward behind the tongue and the hyoid body. The epiglottis stands in front of the laryngeal inlet and bends posteriorly to cover the inlet of the larynx when food is swallowed. The upper anterior surface of the epiglottis is covered by nonkeratinized stratified squamous epithelium. In elderly individuals, the elastic cartilage of the epiglottis is often reduced in size and is replaced by adipose tissue. The vocal cords (folds), which contain striated skeletal muscle and ligaments (mainly elastic fibres), are lined by thin nonkeratinized stratified squamous epithelium, which is firmly attached to the underlying vocal ligaments. The stratified squamous epithelium protects the vocal cords from mechanical stress. The main functions of the vocal cords are to control airflow and facilitate speaking (7).

Oral Premalignant and Malignant Lesions

The World Health Organization (1980) has defined a precancerous lesion as "morphologically altered tissue that is more likely to turn malignant than its apparently normal counterpart." In the oral cavity, lesions such as leukoplakia, erythroplakia, and erythro-leukoplakia are considered to be premalignant.

- Leukoplakia is a white, non-scrapable lesion of the oral mucosa, which cannot be characterized clinically or pathologically as any other disease (8).

- Erythroplakia is a clinical, descriptive term for lesions of the oral mucosa that constitute bright-red, velvety plaque, and which cannot be characterized clinically or pathologically as being due to any other condition (9).

High Risk Sites for Oral Premalignant Lesions

WHO (1980) has suggested that the incidence of the premalignant lesions usually shows a predilection for certain sites: the most common site is the commissures followed by the buccal mucosa, tongue, vermillion border, floor of the mouth and soft palate. Alveolar ridge, hard palate and gingiva form rare sites for leukoplakic lesions. Premalignant lesions associated with smoking usually appear on lips and commissure whereas the lesions associated with chewing habits tend to be limited to areas like the buccal mucosa and hard palate. Unlike a Western population, floor of the mouth leukoplakia is not that common among Asians (10). Erythroplakia and Sublingual keratosis show the highest degree of risk of turning malignant (50% and >40% respectively). The degrees of risk associated with oral precancerous lesions are:

Premalignant lesion	**Risk of malignancy**
Leukoplakia	4%-18%
Erythro-leukoplakia	32%
Oral submucous fibrosis	3%-6%
Lichen planus	1% - 10%

Risk of premalignant lesions depends on:

1. Degree of dysplasia, and
2. Location of the lesion.

Dysplasia is the name given to disordered proliferation of tissue which particularly affects epithelium where there is normally a well-defined pattern of maturation. Dysplasia is characterized by loss of the normal uniformity of individual cells and a tendency for disorganization of the tissue as a whole. It can be a prelude to malignant change (9).

The changes in the epithelium during the development of premalignant lesions can be classified into different degrees of dysplasia (mild, moderate and severe), a significant percentage of which bears a certain risk of malignant transformation.

The histologic features of dysplasia are:

(1) Loss of polarity of the basal cells

(2) Basilar hyperplasia

(3) Altered nuclear cytoplasmic ratio (N/C ratio)

(4) Drop-shaped rete pegs

(5) Irregular epithelial stratification

(6) Abnormal mitoses

(7) Mitotic figures in the superficial layers of the epithelium

(8) Cellular pleomorphism

(9) Nuclear hyperchromatism

(10) Enlarged nucleoli

(11) Loss or reduction of intercellular adhesion

(12) Individual cell keratinization in the spinous layer of cells (11).

Dysplasia can be graded as

- Mild when two of the above-listed features are present,
- Moderate when two-to-four features are present
- Severe when more than 5 of the aforesaid features are present.

Carcinoma-in-situ (CIS) lesions show top-to-bottom dysplastic changes in the epithelium with no signs of invasion into the underlying connective tissue (12). The likelihood of carcinoma developing from dysplastic lesions has been calculated to be 11%, 33% and 56% for cases of dysplasia in the ascending order of its degree respectively, suggesting that the risk of - malignant transformation is directly related to the degree of dysplasia. It is noteworthy that not all dysplastic lesions turn into squamous cell carcinoma and some of them may even show regressive changes (13).

Squamous Cell Carcinoma

The clinical appearance of small, early squamous cell carcinoma of oral cavity may vary from a white, thickened, or verrucous lesion to a velvety plaque or a chronic painless ulcer. More than 90% of SCCs are found to be moderately or well differentiated tumours and metastasis generally occurs in about 80% of the lesions to submandibular or cervical lymph nodes (14). Patients with primary oral carcinoma have a higher risk (4-13%) of developing a second primary, tumour of the upper aerodigestive tract. The prognosis of OSCCs depends on the nature of metastasis.

Aetiology

A variety of local chronic irritations, acting alone or in combination with each other, will produce leukoplakic lesions in many susceptible individuals. The factors which have been suggested to be of etiologic importance in the case of premalignant oral lesions are tobacco (both smoking and chewing types), alcohol, spices (from diet), and genetic susceptibility (15). Other factors like papilloma viruses, ionizing radiation, ultraviolet rays, and such chronic infections such as candidiasis may also play a synergistic role in the process of carcinogenesis. The lesion commences like a protective barrier to prevent further injuries to the underlying tissues and carries a greater risk of malignant transformation, especially in regions such as floor of the mouth where the lining epithelium is that of the non-keratinized type (16). The relative risk for oral cancer increases with duration and quantity of a carcinogen exposure and with the combination of several carcinogens (17).

Genotoxic effects of tobacco smoking and chewing

The most common source of chewing tobacco in several countries in the Asian region is in the betel quid or pan, which is a preparation of betel leaf (Piper betel), areca nut (the fruit of areca palm tree), tobacco, lime and catechu (a resinous extract from the Acacia tree). Sometimes various condiments such as cardamom or cloves are also added in the quids. This is commonly used in northern parts of India (18). In Tamilnadu, a southern state of India, the betel quid does not contain any catechu or spices (Sankaranarayanan et al., 1990). The chewing of betel quid with tobacco has

been established as the principal etiological factor for the high incidence of oral cancer in India and some other South-East Asian countries (19). Tobacco has also been implicated as one of the major etiological factors in the development of other cancers including lung (20) and bladder (21). Potent carcinogenic agents such as tobacco- and areca-nut-specific nitrosamines have been separated from tobacco and areca-nut, the major ingredient of the betel quid (22). Many studies have revealed that the spontaneous generation of reactive oxygen species (ROS) occur during different stages of carcinogenesis (23). There is an increasing body of experimental evidence thatingredients of the chewing mixture such as slaked lime play an important role in the production of reactive oxygen species (ROS) (24). Free radicals such as superoxide anion, hydroxyl anion and hydrogen peroxide anions are highly reactive and may bring in changes such as oxidative damage to cellular organelles and DNA, in turn, leading to genetic damage. This has been experimentally proven by micronuclei test-the presence of micronuclei in exfoliated cells, especially that of the oral cavity (25). Many investigators have shown that there is increased occurrence of micronuclei (26) and/or a higher frequency of sister chromatid exchanges in peripheral lymphocytes in chronic tobacco smokers and betel quid chewers. Tobacco-specific nitrosamines (TSNA), known to be potent carcinogens in experimental animals, may also be causative agents for oral cancers in humans; they may act alone or in combination with other ingredients such as gum catechu and special flavouring agents (e.g., pan masala used in northern parts of India) to bring about the genotoxic effects. Studies have shown that lime together with tobacco and areca nut elevates the pH (becomes highly alkaline) in the oral cavity in betel quid chewers. Lime may

increase the cell turnover by 'killing' the cells and also the likelihood of heritable mutations being transmitted to daughter cells before complete DNA repair (27).

Signs and Symptoms

Small tumors of oral cavity are asymptomatic at the time of presentation. Hence high degree of clinical suspicion is necessary in patients with history of tobacco smoking and betel quid chewing. Most patients present with advanced disease at the time of presentation (28).

Symptoms are generally related to the site of involvement. General symptoms include mucosal growth and ulceration, pain, referred pain to the ear, malodour from the mouth, difficulty with speaking, opening the mouth, chewing, difficulty and pain with swallowing, bleeding, weight loss, change of voice, pain neck, blood in sputum and neck swelling (28).

Site of involvement	**Symptoms**
Buccal mucosa	Ulcer with indurated raised margin
	Exophytic or verrucous growth
Tongue - anterior 2/3rd	Reddish area, nodule, or an ulcer
Floor of mouth	Reddish area, ulcer, papillary growth, discomfort, drooling of saliva
Upper lip	Growth, ulcer
Lower lip	Usually at the vermillion border as crusty induration or ulcer
Gingiva	Ulceroproliferative growth

Alveolar ridge	Difficulty in wearing dentures, loosening of teeth, pain and bleeding
Hard palate	Papillary and exophytic growth
Soft palate/uvula	Ulcer with raised margin or fungating mass
Tonsils	Exophytic growth or ulcer
Base of tongue	Ulcerative mass with induration

Submandibular lymph node is involved in more than two thirds of patients of South Asia with buccal mucosal and gingival cancers. Whereas in patients with cancers of oropharynx, larynx, tongue, floor of mouth from South Asia more than three fourth show neck swellings which implicates that most of these patients have neck node metastasis at the time of presentation.

Diagnostic procedures

Routine radiography for dental involvement

CT imaging is useful for assessing bone involvement

MRI is useful for assessment of soft tissue extension and lymphovascular involvement

Direct pharyngoscopy and indirect laryngoscopy is useful for visualising the lesions of pharynx and larynx

Biopsies from suspicious lesions are taken avoiding areas of necrosis preferably from the edge of the lesions. Patients presenting with neck node enlargement a thorough search for primary lesion is carried out and a biopsy from the primary is preferred.

Fine needle aspiration may be done to assess nodal involvement. When primary could not be identified then FNAC of the node is done. If it is inconclusive then excisional biopsy of the lymph node is done as last resort because this procedure compromises subsequent curative therapy.

Staging

Staging of tumors is done based on the TNM Classification of Malignant Tumours (29)

Spread of tumor

Lymphatic spread

Oral and oropharyngeal cancers with lymph node metastasis generally have worse prognosis. Level II group of nodes are the most common group involved followed by Level I, and Level III. Tumor spread to lymph nodes is predominantly by embolization rather than permeation.

Hematogenous spread

Multiple levels of neck node involvement and extracapsular spread of tumor are predictors of hematogenous spread of tumor. Hemotogenous spread tends to occur via large veins and lung is the most common site of involvement.

Histopathology

The tumour cells show features of squamous differentiation which includes keratinization and formation of squamous pearls and features of invasion into the

surrounding structures with disruption of basement membrane and stromal reaction. They may also show areas of perineural and angiolymphatic spread. Tumours are graded as follows

Well differentiated	resembles closely normal squamous epithelium
Moderately differentiated	less keratinization, nuclear pleomorphism, increased mitoses and abnormal mitotic figures
Poorly differentiated	immature cells with minimal keratinization, typical and atypical mitoses

Most SCCs are moderately differentiated. As such grading has limited prognostic value. But tumors with infiltrative pattern have poor prognosis and the tumors that show expansive growth with pushing margins have better prognosis (28).

Immunohistochemistry

SCCs show positivity for cytokeratins. Low grade tumors tend to show positivity for medium and high molecular weight cytokeratins whereas high grade tumors express positivity for low molecular weight cytokeratins. (30).

Differential diagnosis

Diagnosing squamous cell carcinoma is not problematic usually. Well differentiated SCCs should be distinguished from pseudoepitheliomatous hyperplasia. It is associated with chronic infections like tuberculosis and mycosis, trauma, and granular

cell tumor and lacks cytological features of malignancy. Poorly differentiated SCCs may be difficult to distinguish from other tumors like melanoma, adenocarcinoma or neuroendocrine tumor where immunohistochemistry could be used to arrive at the diagnosis (28).

VARIANTS OF SQUAMOUS CELL CARCINOMA

Verrucous carcinoma is a well differentiated SCC with very low risk of metastasis. It presents as a slow growing exophytic warty growth. These tumors are characterised by thickened club shaped papillae with pushing margins of stromal invasion. Well differentiated squamous epithelium with hyperkeratosis and the cells are larger than SCC cells but lacks cytological features of malignancy (31).

Basaloid squamous cell carcinoma is an aggressive high grade variant of SCC. It is characterised by two types of cells basaloid cells and squamous cells. The basaloid cells are small cells with scant cytoplasm, hyperchromatic nuclei and no nucleoli which are arranged closely in lobular pattern with areas of comedo type of necrosis and cystic spaces which are Periodic acid Schiff (PAS) negative and Alcian blue positive. The basaloid cells and squamous cells are found adjacent to one another (32).

Papillary squamous cell carcinoma presents with an exophytic papillary growth and has a good prognosis. The cells are arranged in a papillary growth pattern with thin fibrovascular core covered by basaloid squamous cells with areas of stromal invasion and adjacent lymphoplasmacytic infiltrates (33).

Spindle cell carcinoma is a biphasic tumor composed of squamous cell and spindle cell component. The spindle cell component forms the majority of the tumor mass, which have a mesenchymal appearance but of epithelial origin. The spindle cell component most commonly resembles fibrosarcoma or malignant fibrous histiocytoma (34). These cells may also show areas of osteosarcomatous, chondrosarcomatous or rhabdomyosarcomatous differentiation. Squamous cell component is usually present in most cases either as invasive carcinoma or in situ carcinoma. Sometimes where only spindle cell component are seen extensive tissue sections are needed and immunohistochemistry can be used for differentiation (35).

Acantholytic squamous cell carcinoma is an uncommon variant characterised by cells arranged in nests with areas of acantholysis of tumor cells creating a pseudolumina which gives an appearance of glandular differentiation. The pseudolumina consists of tumor cell debris but negative for mucin production. Adjacent stroma shows lymphoplasmacytic infiltration (36).

Adenosquamous variant of squamous cell carcinoma is a rare aggressive neoplasm with both squamous and adenocarcinoma components. The squamous component is either invasive or in situ carcinoma (37). The adenocarcinoma component is present in the deeper parts of the tumor where the cells are arranged in tubular pattern with areas of glands in glands appearance. Mucin is found within the glandular lumen and intracellular as well.

Lymphoepithelial carcinoma is a poorly differentiated variant of squamous cell carcinoma with prominent lymphoplasmacytic infiltrates and resembles

nasopharyngeal carcinoma morphologically. The tumor cells are arranged in island and sheets surrounded by desmoplastic stroma. The cells have moderate eosinophilic cytoplasm indistinct cell borders with monomorphous vesicular round to oval nuclei and prominent nucleoli. The tumor is infiltrated by lymphocytes and plasma cells (38). Immunohistochemically the cells show positivity for epithelial cell markers cytokeratin and epithelial membrane antigen. It is associated with Epstein Barr virus(EBV) infection which show positivity for EBV encoded RNA (EBER) (39).

Other tumors that occur in oral cavityinclude squamous papillomas, granular cell tumor, salivary gland tumors, soft tissue tumors, hematolymphoid tumours, melanomas and metastatic tumors.

Proto-oncogenes, Oncogenes and Suppressor genes

Several genetic alterations that perturb normal cellular growth and developmental control mechanisms can lead to abnormal proliferation and differentiation (40). These include point mutations, deletions, translocations, amplifications and gene rearrangements, and occur primarily in two classes of genes, namely oncogenes and tumour suppressor genes. While mutation or amplification of certain genes can lead to abnormal cellular proliferation, loss or mutation of tumour suppressor genes (which normally suppress abnormal proliferation), can activate neoplastic transformation (41). Proto-oncogenes (cellular oncogenes: c-onc) are present in normal cells, controlling cell proliferation and differentiation. Oncogenes are closely related to proto-oncogenes, but they have mutated to produce abnormal protein products or to allow gene over-expression leading to a loss of the normal constraints on their activity

(42). The genes which act by regulating the oncogenes or by producing substances that antagonize the actions of oncoproteins, are termed tumor suppressor genes (emerogenes, anti-oncogenes, or recessive cancer susceptibility genes) (43). Mutations in tumor suppressor gene p53 are considered to be one of the salient features of a large spectrum of human malignancies. Multiple oncogenes may be involved in oral carcinoma, especially c-myc with H-ras explained by Field and Spandidos in 1987(44) or c-erb-B-1 explained by Yarnada et al in 1989 and also by Saranath et al in 1992 (45).

Molecular Lesions in Oral Cancer

A mutation is a change in the nucleotide sequence of the DNA molecule which may occur within a gene or in the intergenic regions. If a mutation occurs in an intergenic region it will probably be silent and have no detrimental effect on the cell. But when the mutation occurs in a gene, it may alter the gene product and generate observable changes in the organism which is referred to as a change in phenotype. Mutation can happen at the level of DNA sequences (point mutation, insertion, deletion and inversion) or at the level of gene. The function of a gene can be blocked at the protein level by altered gene(s) which encodes a mutant product capable of inhibiting the wild-type gene product in a cell. Such a mutation would be 'dominant negative' because its phenotype is manifested in the presence of the wild-type gene, but it inactivates the wild type activity. Herskowitz (1987) referred to this kind of mutations as "antimorphs, 'antagonistic mutant genes' having an effect virtually contrary to that

of the gene from which they were derived (46)." Basically, the process involves the inhibition of function of a wild-type gene product by an overproduced inhibitory variant of the same product. If a protein has multiple functional sites (e.g., multimeric nature), a derivative capable of interacting with wild-type polypeptide but otherwise defective will be inhibitory, especially if it forms multimeric forms which are non-functional. Hence, dominant negative mutant proteins will retain an intact functional subset of the domains of the parent wild-type protein, but have the complement of this subset either missing or altered so as to be non-functional (47). The dominant negative function is achieved by the formation of inactive complexes (hetero-oligomers) between the mutant and wild-type p53 proteins (48), and this may, in turn, affect the sequence specific DNA-binding activity of p53 (49). Mutation in one of the two alleles of the gene that act in a dominant manner over the normal phenotype (wild-type) of allele leads to a gain of function, or uncontrolled proliferation. Mutation of one of the allele followed by loss of, or a reduction to homozygosity in the second allele will lead to a loss of regulatory effect on cell proliferation (50). p53 oncoprotein appears to block the progression of cells through the late presynthetic phase of replication: mutant forms may fail to maintain this control or may even stimulate cell proliferation (51). The most frequently documented genetic change in human oral cancer is in the short arm of chromosome 17 (17p), in the region of the suppressor gene p53. Mutations or deletions in onco-suppressor genes may give rise to defective suppressor proteins thereby leading to the loss of tumour suppressor function. Such genetic markers as p53 may play an important role in the development of oral squamous cell cancers, and give some insight into the molecular mechanisms that are

responsible for neoplastic transformation (52). Mitogenic agents such as epidermal growth factor produce pleiotropic response involving the activation of various biochemical processes that are responsible for cellular growth and development. The interaction of the growth factor and its effects on oral mucosa will be described in subsequent sections

Mitogen-Activated Protein Kinases (MAP kinases)

The growth signals obtained at the cell surface are transduced to the nucleus through a cascade of tightly regulated phosphorylation events mediated by kinases such as protein kinase-C (PKC), casein kinase and mitogen activated kinases (MAPK). Recent studies have shown that the ligands that bind to epidermal growth receptor may also act through the receptor-specific threonine kinases (53). There is an increasing body of evidence which suggests that these kinases play an active role in the cellular proliferation and/or differentiation (54). Since many membraneassociated growth factor receptors have been shown to exhibit ligand-dependent proteintyrosine kinase activity, it is thought that tyrosine phosphorylation is an early event in the propagation of signal transduction cascade. Furthermore, oncogenes that are improperly regulated have been shown to induce cellular transformation via aberrant phosphorylation of tyrosine residues (55). An important kinase among these enzymes, originally named microtubule-associated protein kinase-2 (MAP-2) because it phosphorylates microtubule-associated protein, is now referred to as mitogenactivated protein kinases (MAP kinase) (56). The MAP kinase (MAPK) requires both tyrosine and threonine phosphorylation for activity and recent studies has revealed that MAP kinase is not a

single enzyme, but a family of kinases that have been to referred to as Extracellular signal Regulated Kinases (ERKs) (57). Depending upon the in vitro substrates that have been used to characterize these kinases, they are again subdivided into myelin basic protein kinases or MBP, ribosomal S6 protein kinase-kinases (RSKs) and EGF-receptor threonine kinases or ERK (58). The role of MAPK in signal transduction pathways may be summarized as follows: Signals initiated by receptor tyrosine kinases (RTKs), by protein kinase-C or by signal transducing G-proteins rapidly converge and activate the cytoplasmic MAPK via phosphorylation of tyrosine and threonine. The activated MAPK then activate the RSKs by serine threonine phosphorylation, which helps the RSKs to phosphorylate their targets such as ribosomal S6 protein. One of the MAPKs, epidermal growth factor receptor-tyrosine kinase (ERT kinase) is proposed to down-regulate the activity of EGFR soon after ligand binding while RSK has the potential to turn off a variety of signal transducers that are modulated by tyrosine- or serine threonine phosphorylation via other kinases that may, in turn, activate RSKs (59). Many extracellular stimuli that influence cell proliferation and differentiation, including growth factors, cytokines and hormones can stimulate MAP kinases and the activation of MAPKs require phosphorylation of tyrosine and threonine residues. Studies concerning the mechanism of activation of MAPKs are in progress now (60).

Tumour Markers

Neoplastic transformation is a multistage process which involves a number of aberrant genetic events that are progressively associated with the acquisition of a state of malignancy. Many oral cancers are known to arise from premalignant lesions. The substances (eg, oncoproteins, enzymes, growth factor receptors), that are used to study the changes in preneoplastic as well as neoplastic tissues, are referred to as tumour markers (61). A number of growth factors such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) and -beta (TGF-β) show altered expression, or disregulated production during the process of carcinogenesis (62). An increased production of growth factors may indicate the presence of abnormal growth or some disturbances in cellular proliferation. Altered proliferation or differentiation may also be induced by deregulation of tumour suppressor genes such as p53 (63).

Onco-suppressor gene p53

p53, a proto-oncogene product, is a cellular protein expressed in low levels in non-transformed cells. On the other hand, tumour-derived cells and transformed cell lines show a many-fold increase in the expression of p53 (64). Unlike the proteins of non-transformed cells, the mutant protein is likely to form complexes with transforming proteins of viruses such as Simian Vacuolating virus (SV40) leading to the acquisition of a stable conformation than the wild-type protein. The results of several studies have

supported the view that increased levels of p53 effect changes in the phenotype of normal cell and that over-expression of the protein leads to the immortalization of cell lines. It is suggested that over-expression of p53 is a common event in the multistep carcinogenesis in colo-rectal carcinomas and oral squamous cell carcinomas (65).

Functions of p53

Finlay et al. (1988) have demonstrated that the wild-type sequence of the proto-oncogene does not code for a transforming protein, but loss of function or mutation in the gene possibly transactivates the protein culminating in malignant transformation. The p53 protein appears to block the progression of cells through the late G1 phase of replication: mutant forms fail to perform this function and may even stimulate cell proliferation (66). A clear relationship has been established between p53 expression and heavy smoking and alcohol consumption and the protein appears to play a central role in oral carcinogenesis, possibly being mutated by carcinogens in tobacco and alcohol (67). Studies using ‘high-tech’ devices have revealed that p53 has a possible role in the control of mRNA synthesis and/or maturation (68). Analysis of non-transformed cells, non-SV40-transformed cells, and growth-arrested and –stimulated mouse cells have revealed that the p53 plays an important role in the cell cycle and the subcellular distribution of the protein varies throughout the cell cycle. p53 forms high molecular weight complexes with E6 proteins of the human papilloma virus types 16 and 18), heat shock proteins and protein kinases (69). Since p53 is a nuclear protein which seems to be involved in the regulation of cell cycle, especially DNA replication

and transcription, the mutant variety of the protein shows a relatively high affinity for binding to single stranded DNA than to double-stranded DNA. Although this is true, the wild-type protein p53is more efficient in binding SV40 DNA fragment which consists of the sequences known to regulate the activity of SV40 promoters. Hence, it is possible that the protein plays a dual role by involving in DNA replication and in the regulation of gene expression (61).

The p53 tumour suppressor gene is a frequent target for structural alterations in a large number of human malignancies. The levels of wild-type p53 increase in response to DNA-damaging agents such as ionizing radiation, leading to G1 arrest. Because of high levels, p53 may transcriptionally induce the expression of the growth arrest and DNA damage protein, GADD45, resulting in the inhibition of progression from GI into S phase (62). Over-expression of wild-type p53 in transformed cells can arrest cell - proliferation, reverse a tumourigenic type, and sometimes induce apoptosis or differentiation. The wild-type p53 has been shown to be capable of attaching to specific DNA sequences thereby acting as a transcriptional modulator. Furthermore, over-expression of wild-type p53 represses transcription from a variety of promoters probably through basal transcription factors. Studies have revealed the existence of a p53-binding protein, which can associate with both wild and mutant types of the protein (63). Momand et al. (1992) have identified this protein as the product of murine double minute (MDM2), a putative proto-oncogene, over-expression of which interferes with sequence-specific transcriptional activation by wild-type p53. Over-expression of wild-type p53 is capable of sequencespecific binding to a region within the mdm2 gene and of transactivating directly this gene (64). High levels of Mouse

double minute homolog-2 (MDM2) protein, similar to the DNA tumour virus oncoproteins may inactivate the tumour suppressor activity of p53 by complexing to it (65). It is also possible that the increased levels of wild-type p53 may transactivate EGF receptor promoter sequences and that there exists a common regulatory mechanism to control cell proliferation mediated through both p53 and EGF receptor (66).Mutations that activate transformation may be the ones that result in a loss of function of the wild-type p53. Finlay et al. (1989) suggested that over-expression of p53 mutants enhances the transformation process by the formation of non-functional multimeric complexes which can inactivate wild-type of the protein thereby giving a growth advantage over the other cells. Since the p53 acts as a tumour suppressor gene, it possibly gets involved in the negative control of growth and differentiation via its protein product (67). This shows that the interaction of mutant forms of the oncoprotein with that of the wild-type would switch off the activity of the latter or considerably decrease its endogenous tumour suppressor activity (dominant negative); thus, making the cell to be deficient in the function of that gene product. There is also evidence that at least some mutant p53 proteins may also play a key role in allowing cells to enter the S-phase which fails to correct DNA damage. In other words, overexpression of the mutant p53 and its interaction with that of the wild-type protein makes the cell more susceptible to uncontrolled proliferation (68). It seems that p53 mutation is an important step for cell transformation in vivo.

p53 Expression in other areas

Although the expression of proto-oncogene product p53 is very low in normal non-transformed cells, most of the neoplastically transformed cell lines show a many-fold increase in the levels of the oncoprotein. Mutations of p53 gene have been reported in most of malignant tumours: breast, liver, lung,colo-rectum and bladder (65).

p53 and Oral Cavity Lesions

Heavy smoking and drinking or tobacco smoking coupled with tobacco/betel quid chewing are the major parameters which correlate with the over-expression of oncoprotein p53 in patients with Oral cavity squamous cell carcinoma. The high percentage of p53 positivity, in heavy smokers, may be due to accumulation of chemical carcinogens in the tissues over time. Field et al. (1991) suggested that the excess carcinogen may damage the integrity of the gene leading to its over-expression and that there might be a loss of suppressor activity with a gain in the dominant transforming activity. It has been established that G→T transversions are the most frequent p53 mutations in non-small cell lung cancer and in oesophageal squamous cell carcinomas. It is worth noting that benzopyrene, a major carcinogen in tobacco smoke, is known to induce G→T transversions. This strongly suggests that tobacco use, a known risk factor in oral squamous cell cancer, is linked with the mutations that occur in the gene on prolonged exposure to tobacco smoke (69). A number of publications have shown that there is over-expression of p53 oncoprotein in oral

SCCs. The percentage of oral SCCs positive for p53 varies from 35% to 93.5%, with most of the reports showing a positive reaction in more than 50% of the cases. The average percentage of p53 staining in oral SCCs is 60.5. However, a recent paper by Ranasinghe et al. (1993) showed a very low percentage (11%) of oral SCCs that were positive for p53 from a South Asian population. The sharp contrast of the result from this study as compared with those of other studies raises the possibility that there might have been technical problems in the staining procedures. On the other hand, it is well known that the etiological factors for oral malignancies in the South Asian population are different from those of the Western World (70). In Western countries, oral SCC only accounts for 5% of all the cancers and the main etiological factors are smoking, and alcohol or a combination of the two. In the South Asian population, oral SCCs accounts for more than 40% of all cancers (71) and the main etiological factors are betel quid with tobacco chewing and tobacco smoking (72). It is, therefore, possible that the low percentage of p53 expression in oral SCCs from the South Asian population by Ranasinghe and his associates (1993) may reflect the differences in the etiological factors when compared to other parts of the world. It is likely that a large sample containing more SCC cases might draw more reliable conclusions. Further studies are needed to give more insight into the functional aspects of p53 in oral squamous cell carcinomas.

Overview of Immunohistochemistry

Immunohistochemistry is a combination of anatomical, immunological and biochemical technique. It identifies the specific components of the cells by interaction

of the target antigens with visually labelled antibodies. It helps to visualise the distribution and localisation individual cell components within the cells. There are many approaches and methodology in IHC but they are broadly divided into two groups i.e., sample preparation and labelling.

Introduction - History

Even though the principle of IHC has been known since 1930, only in 1942 Coons et al reported the first IHC study. He identified pneumococcal antigens in the infected tissue by using fluorescein isothiocyanate labelled antibodies. Later on many improvements and modifications has been made in the field of protein conjugation label detection, tissue fixation, antigen retrieval and light microscopy. Now IHC has become a routine tool in diagnostic and research laboratories.

Applications

IHC is used for diagnosing diseases, development and assessment of drugs and in biological research activities. In pathology IHC is used as a diagnostic tool to identify benign and malignant tumors, staging and grading of tumors and helps in identifying the cell of origin in metastatic tumors.

Individual tissue samples are prepared on the slides, processed and stained and visualised. Tissue microarray techniques allow multiple samples to be prepared on a single slide for comparative analysis and is also cost effective and less time consuming. Automated techniques are available for sample preparation and staining. The samples are visualised by either light microscopy or fluorescent microscopy.

Recent advances in technology have enabled in high quality image capturing and image analysis and quantification of multiple IHC parameters.

Sample preparation

Sample preparation is a critical step in IHC as it helps in maintaining the cell morphology, tissue architecture and preserving the antigenicity of the target epitopes.

Tissue Collection

Fresh tissues are preferred for IHC but it can also be done on formalin fixed tissues. Biopsies are generally preserved in formalin immediately and sent for IHC analysis. Tissues admixed with blood are washed with water prior to fixation as hematologic antigens may interfere in detection of target antigens.

Tissue Fixation

Tissues are fixed chemically either by cross linkage of proteins or by denaturation of proteins rendering them insoluble. Prolonged fixation or improper fixation can cause masking of the target antigens so right fixation method has to employed based on the target antigen to be analysed.

Formaldehyde is the most common fixative used. It is a semi-reversible, covalent crosslinking reagent that allows tissues to be immersed or perfused for prolonged period of time depending on requirements.

Tissue Embedding

In order to maintain the natural shape and architecture of samples for long term storage and thin sectioning the tissue samples are embedded in paraffin wax. Certain samples that are too sensitive for chemical fixation or which could be removed during the process of dewaxing are embedded in cryogenic medium and then snap frozen in liquid nitrogen.

Sectioning and Mounting

The decision to section tissue is dependent on the application to be used. For whole mount IHC when the samples are of less than 5mm thickness sectioning is not required. Samples that require multiple staining procedures sectioning is needed.

Formalin fixed tissues that are embedded in formalin are sectioned into thin slices of 4 to 5µm thickness using a microtome. These sections are then mounted on the adhesive coated or positively charged slides. The commonly used adhesives are 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine. Other physical adhesives like gelatin, egg albumin or Elmer's glue are also used. After mounting the sections the slides are dried in the oven or microwave before proceeding to deparaffinization.

The frozen section are cut using a pre-cooled cryostat and mounted onto the adhesive glass slides, followed by overnight drying at room temperature and fixed with pre-cooled -20°C acetone. Depending on the target antigens this step may be skipped.

Tissue section must be deparaffinised before staining because wax interferes with the penetration of the reagents. For the labelled antibodies to specifically bind to the

correct epitopes the formalin fixed tissues must be also pre-treated to unmask the antigens.

Deparaffinizing (Clearing) and Rehydrating

Xylene is miscible with paraffin and alcohol and is most commonly used to remove paraffin from the tissue sections. The slides left in three changes of xylene for 5 to 10 minutes. After clearing the sections are rehydrated in decreasing grades of alcohol with water starting with absolute alcohol 10 minutes each. The sections are then taken to distilled water wash. The number of washes and the reagent concentration are standardized according to the laboratories and optimized for antigens and tissue samples accordingly. To avoid non-specific antibody staining and high background staining the section should never be allowed to dry before mounting.

Antigen Retrieval

Formaldehyde forms methylene bridges between the proteins which in turn interferes with the epitope recognition of the primary antibodies. These methylene bridges are removed by two methods.

Heat induced epitope retrieval (HIER)

This is most commonly used method for antigen retrieval. Microwave oven, pressure cooker or vegetable steamer is used as the heating sources with buffers such as Sodium citrate or Tris/EDTA. The temperatures, pH of the buffer and incubation time are the critical factors involved in this method.

Proteolytic induced epitope retrieval (PIER)

Proteolytic enzymes such as pronase, pepsin, ficin, trypsin or proteinase K are used to partially digest the proteins and unmask the antibody epitopes. The proteolytic enzyme concentration and the incubation time are the critical factor involved in this method. Both methods can either be used separately or I combination. In alcohol fixed tissue samples antigen retrieval increases the antigenicity of the samples.

Blocking Endogenous Targets for IHC

Immunohistochemistry uses reagents like peroxidases and biotin, which are also found in tissues and these, may cross react with antibody and interfere with the antigen detection. This may lead to false positive results. Hence before primary antibody is added to tissue sections these endogenous factors are blocked so they do not interfere with antibody epitope detection.

Peroxidases

Horse radish peroxidase is the most commonly used reporter for target antigen detection which is coupled with 3,3-diaminobenzindine (DAB) chromogen. Endogenous peroxidases and pseudoperoxidases present normally within the tissues reacts with hydrogen peroxide to reduce DAB chromogen and resulting in non-specific staining of tissues.

This is circumvented by using 3% hydrogen peroxide in methanol or water which supresses the endogenous peroxidase activity if there is any, after adding primary antibody. Endogenous peroxidase activity in tissue sections can be assessed by

treating the formalin fixed tissues directly with peroxidase substrate such as DAB, and formation of colored precipitate will indicate the presence of endogenous peroxidase activity.In tissues that are rich in endogenous peroxidase activity alternatively calf intestine alkaline phosphatase can be used as enzyme label.

Endogenous peroxidase block is done after adding the primary antibody and before adding the peroxidase conjugate so that it does not interfere with the immunoreactivity of the primary antibody.

Blocking nonspecific sites

Antibodies generally bind to specific epitopes. But they may also bind weakly or partially to sites non-specific proteins also known as reactive sites that are similar to the cognate binding sites of the target antigen. This leads to non-specific background staining which in turn interferes with the target antigen detection. Buffers such as normal serum, non-fat dry milk, Bovine serum albumin (BSA) or gelatin and other commercial buffers blocks the reactive sites. Blocked reactive sites do not interfere with primary or secondary antibody and thus reducing the background staining.

Sample labelling – Immunodetection

Detecting the target antigen with antibodies is a multistep process and it requires optimization at every level to maximise the signal detection. Both primary and secondary antibodies are diluted in the buffer. This helps in uniform dissemination of antibody throughout the sample, stabilisation, and reduces the non-specific background staining. The diluent used is optimized for the specific antibody used.

In between antibody application the section are rinsed in rinse buffers which remove the unbound antibodies and weakly bound antibodies to the non-specific reactive sites and thus reducing the background staining. Rinse buffers are usually simple solution.

Antibody-mediated antigen detection approaches are separated into direct and indirect methods.

Direct method

The primary antibody is directly coupled with the conjugate which can either be a flourochrome or an enzyme. The labelled primary antibody reacts with the antigen directly. The disadvantage of this method is that there is no antigenic signal amplification.

Indirect method

The antigenic sites on the primary antibody react with the labelled secondary antibody. Horse radish peroxidase is the most commonly used label together with chromogen substrate. This method allows for antigenic signal amplification as many secondary antibodies can react with different antigenic sites of a single primary antibody and same secondary antibody can be used against different primary antibodies of same species.

In Polymer chain indirect method the secondary antibody is conjugated with peroxidase enzyme which is labelled with a polymer such as dextran chain. Dextran chain polymer has up to 70 enzymes and 10 secondary antibodies. This method is

quick, easily reproducible and reliable. This is the most common method used in diagnostic laboratories.

The target antigens are detected either by chromogenic method or fluorescent method. For fluorescent detection the primary or secondary antibody is conjugated with a flourophore which is then detected by fluorescent microscopy. Chromogenic method employs enzymes such as horse radish peroxidase or alkaline phosphatase which produces insoluable colored precipitates on reaction with DAB or nitro-blue tetrazolium(NBT) and 5-bromo-4-chloro-3'-indolyphosphate(BCIP) that can be detected by light microscopy.

Chromogenic counterstains

Hematoxylin is most commonly used. Hematoxylin on oxidation combines with aluminium ions to form an active metal dye complex which on binding with the lysine residues within the histones of mammalian dna produces dark blue staining of the nucleus. Nuclear fast red reacts with the nucleic acids resulting in red nucleus. Methyl green stains the nucleus green.

Fluorescent Counterstains

DAPI (4', 6-diamino-2-phenylindole) and Hoechst are common nuclear dyes used for fluorescent IHC.

Sealing the Stained Sample

After all staining is completed; the sample should be preserved for long-term usage and storage and to prevent enzymatic product solubilisation or fluorophorephoto

bleaching. Sealing the sample by mounting a coverslip with an appropriate mountant stabilizes the tissue sample and stain. An antifade reagent should also be included if fluorescent detection will be performed to prolong fluorescence excitation. The coverslip can then be sealed with clear nail polish or a commercial sealant after the mountant has cured to prevent sample damage.

Sample Visualization

Once the sections are prepared, the samples are viewed by light or fluorescent microscopy, depending on the antibody detection method.

MATERIALS AND METHODS

MATERIALS AND METHODS

The present study is a retrospective study. Biopsies from ENT and Dental department Tirunelveli medical college received for routine histopathological evaluation in the department of Pathology, Tirunelveli medical college during the study period were taken for this study. This study comprises 60 specimens received during November 2014 till May 2015.

The habitual history is obtained from histopathological requisition form and case history from case sheet

Methods of study:

- Complete clinical history of the patients presenting with leukoplakia, erythroplakia, mucosal ulcer, growth, or pain in the upper aero digestive tract.
- Complete personal history of patients with details of smoking history, betel nut chewing, pan or gutkha taking habit including total duration, frequency per day and amount of consumption per day.
- Histopathological evaluation of the specimens of hematoxylin and eosin stained slides.
- p53 immunostaining and evaluation of the specimens.

Sample size:

60 cases of Oral cavity lesions from November 2104 till may 2015

Sample design:

Purposive sampling

Inclusion criteria

- Cases received for histopathological evaluation at Department of Pathology Tirunelveli Medical College, Tirunelveli.

Exclusion criteria

- Specimens not sent in formalin
- Specimens with inadequate history

Study period

From 2014 to 2015

Place of study

Department Of Pathology

Tirunelveli Medical College

Tirunelveli

Brief procedure:

Demographic profile, relevant clinical history like age, sex, habitual history etc. were recorded for all patients from the clinical case records.

The received biopsy specimens are subjected to fixation in 10% neutral buffered formalin .After adequate fixation (usually 24 hours) surgical grossing was done according to the standard protocol and a detailed gross description was made. Extensive sampling is done to search for suspicious areas and then tissue sections are taken and subjected to routine manual tissue processing and paraffin embedding. Sections of 4-5 µ thickness were taken and routinely stained with haematoxylin and eosin (H & E) and mounted with DPX mountant .The slides are examined under a light microscope. Cases are grouped as non-malignant, premalignant and malignant.

Cases of squamous cell carcinoma were graded based on histomorphological analysis as

- Well differentiated,
- Moderately differentiated
- Poorly differentiated

All these cases are further subjected for immunohistochemical analysis with p53.

IHC Procedure:

APES coated slides

- Amino propyl triethoxy saline (APTS) - 2ml
- Acetone - 98ml

Acid clean slides are kept in acetone for 2 minutes, transferred to 2% APTS for 2 minutes then finally transferred to acetone for 30 seconds. Allow to air dry and keep the slides in dust free box. These slides are used for taking sections from paraffin blocks.

- Incubate the slides in incubator for 1 hour at 55°C temperature.
- Deparaffinise the sections in 3 changes of xylene 5 minutes each.
- Hydrate the sections in decreasing grades of alcohol from absolute alcohol, to 90%, and 70% of alcohol. Bring the sections to water.

Antigen retrieval solution

- Citrate bath (pH 6.0)
- Tris sodium citrate 2.96gms
- Distilled water 1000ml

Slides are kept in boiling solution in cooker for 20 minutes at 100°C.

Tris buffered saline (TBS) pH 7.4 to 7.6

- Sodium chloride 20gms
- Tris 1.512 grams

- Distilled water 2500ml

Adjust pH with 1N HCL (Concentrated HCL – 10 ml and Distilled water - 90ml)

Staining procedure

Super sensitive polymer-HRP detection system is used and moisture tray is used for staining

- Apply peroxide block to the specimen according to tissue size and incubate for 10 minutes at room temperature.
- Wash in TBS twice, each for 5 minutes, drain and blot gently around the section.
- Apply power block and incubate for 10 minutes at room temperature.
- Without washing apply appropriate primary antibody and incubate for 30 minutes at room temperature (20 degrees to 22 degree Celsius).
- Wash in TBS twice each 5 minutes, drain and blot gently around the section.
- Apply Polymer-HRP reagent and incubate for 30 minutes at room temperature (20 degrees to 22 degree Celsius).
- Wash in TBS twice each 5 minutes, drain and blot gently around the section.
- Apply working chromogen substrate solution and incubate for 10 minutes at room temperature (20 degrees to 22 degree Celsius) or until acceptable color intensity has been reached.
- Wash with TBS buffer followed by distilled water.
- Counter stain in hematoxylin for 15 – 30 seconds.
- Wash with distilled water – 2 changes

- Dry, clear and mount with DPX mountant.

Development of brown color indicates positive staining.

Habitual history:

Tobacco smoking and betel quid chewing history is collected from the histopathological request and from the clinical data of case sheets. These cases are further subdivided into

Group 0 – non-smokers with no history of smoking

Group 1 –history of smoking 1 to 5 cigarettes or beedis per day

Group 2 – history of smoking 6 to 10 cigarettes per day

Group 3 – history of smoking more than 10 cigarettes per day

Similarly betel quid chewers are grouped as

Group 0 – no history of chewing tobacco

Group 1 – history of chewing 1 to 5 betel quids per day

Group 2 – history of chewing 6 to 10 betel quids per day

Group 3 – history ofchewing more than 10 betel quids per day

Statistical analysis:

The intensity of p53 oncoprotein staining reaction was graded as follows:

negative or equivocal -

weakly positive +

moderately positive ++

strongly positive +++

The pattern of the staining for p53 positive cells was classified according to the number of positive cells in a given field; scattered when only a few positive single cells were seen in a given field, and diffuse when a large number of positive cells were clumped together in a given area (65). Fischer's exact test of significance is performed to examine the association between staining intensity (measured on a 4-point scale) and degree of malignancy. To analyze the correlation between the expression of p53 oncoprotein and the chewing habits (frequent chewers Vs infrequent chewers), the Fisher Exact test of significance was used.

RESULTS

RESULTS

Table - 1. **Tobacco Chewing habits (Betel quid/Pan masala/Gutkha).**

Tobacco Chewing Habits	No. of Cases
Non Chewers	8
Chewers	52
Total	60

Table - 2. **Smoking habits.**

Smoking Habits	No. of Cases
Non-Smoker	25
Smoker	35
Grand Total	60

Chart - 1

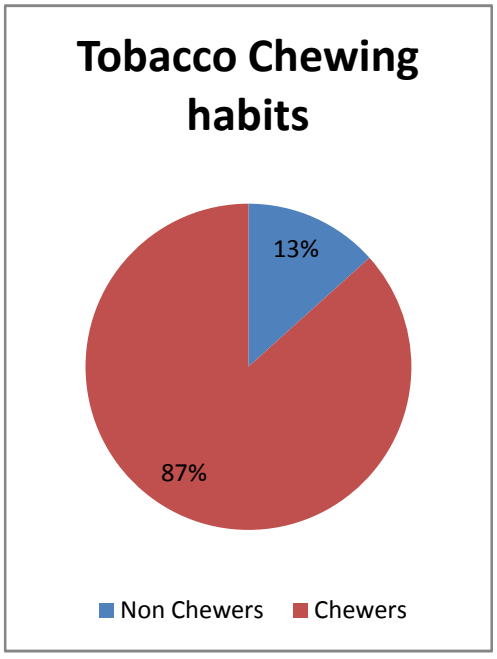
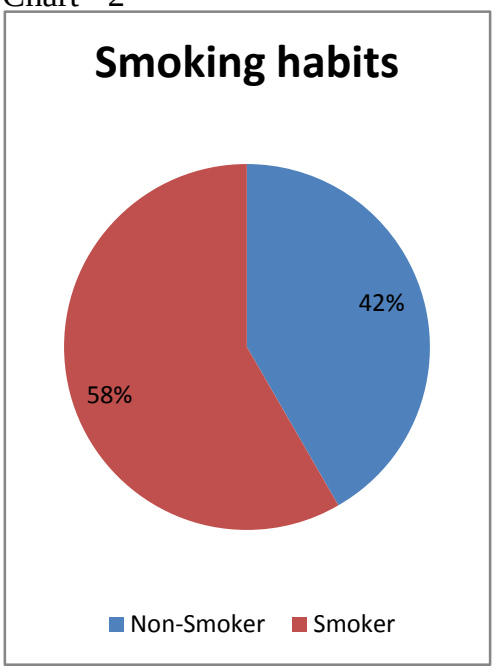


Chart - 2

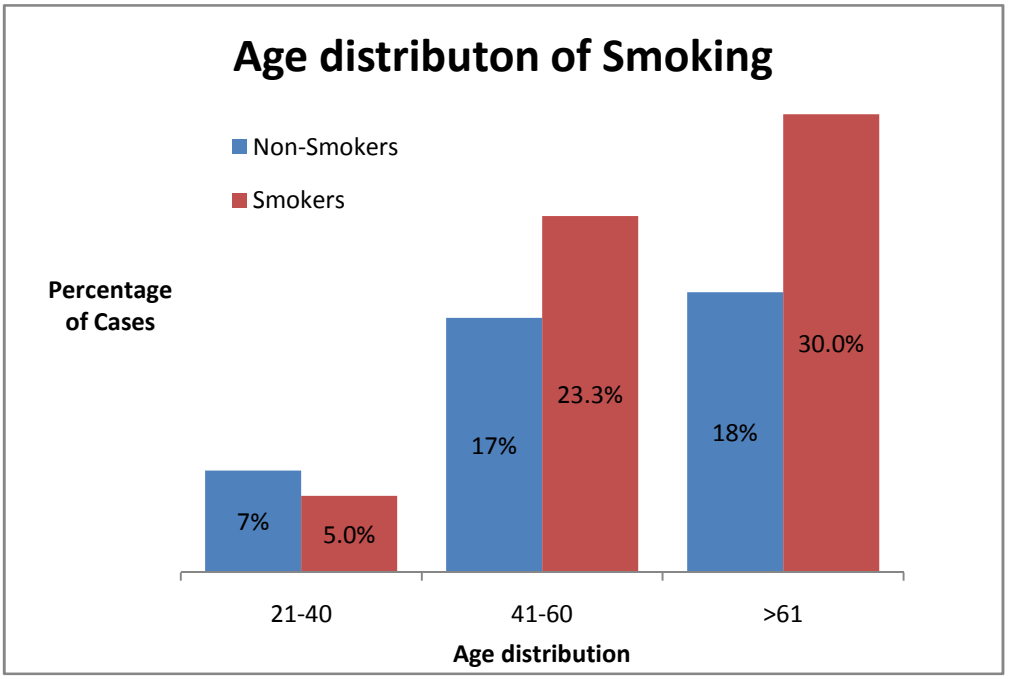


The above charts and tables show that the percentage of non-smokers is high compared to the percentage of non-chewers of tobacco. It is partly due to the fact that none of the female cases in the study group had a history of smoking habit. But betel quid chewing is much more prevalent among females.

Table - 3. **Smoking habits among age groups**

Age Distribution	Smoking Habits		Total
	Non-Smokers	Smokers	
21-40	4	3	7
41-60	10	14	24
>61	11	18	29
Total	25	35	60

Chart -3

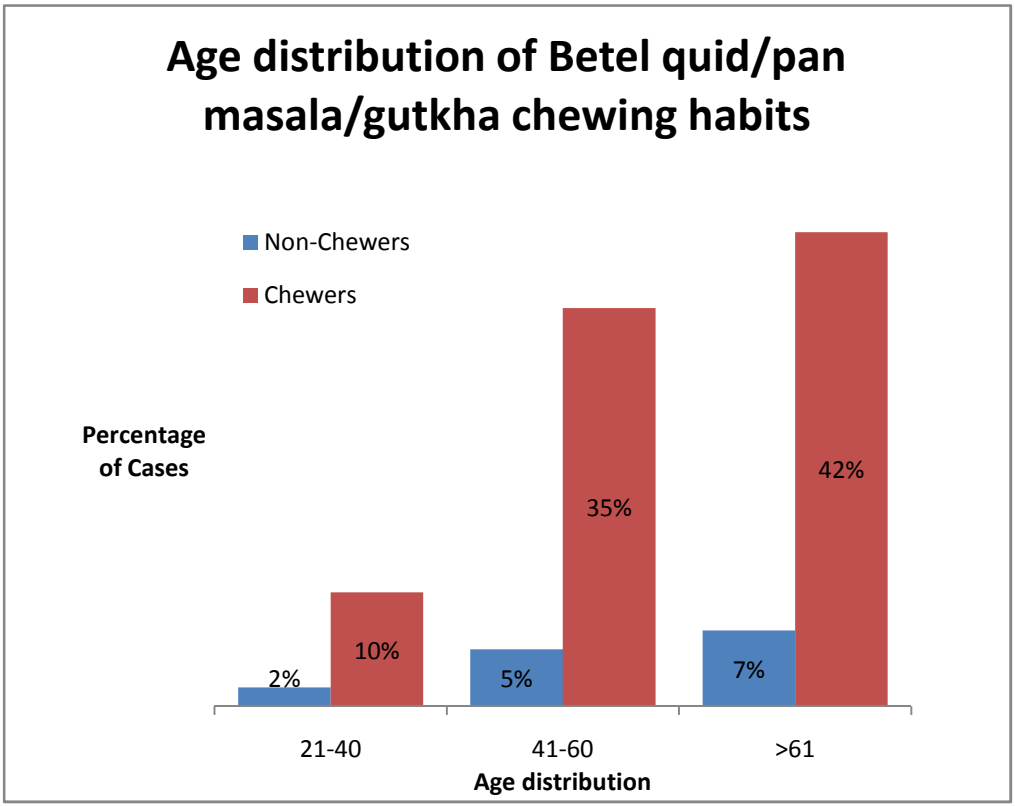


The habit of smoking is more in the older age groups than in the younger adults within the study population

Table - 4. **Tobacco Chewing (Betel quid, Pan masala, Gutkha) habits among the age group**

Age groups	Tobacco chewing habits		Total
	Non-Chewers	Chewers	
21-40	1	6	7
41-60	3	21	24
>61	4	25	29
Total	8	52	60

Chart - 4.

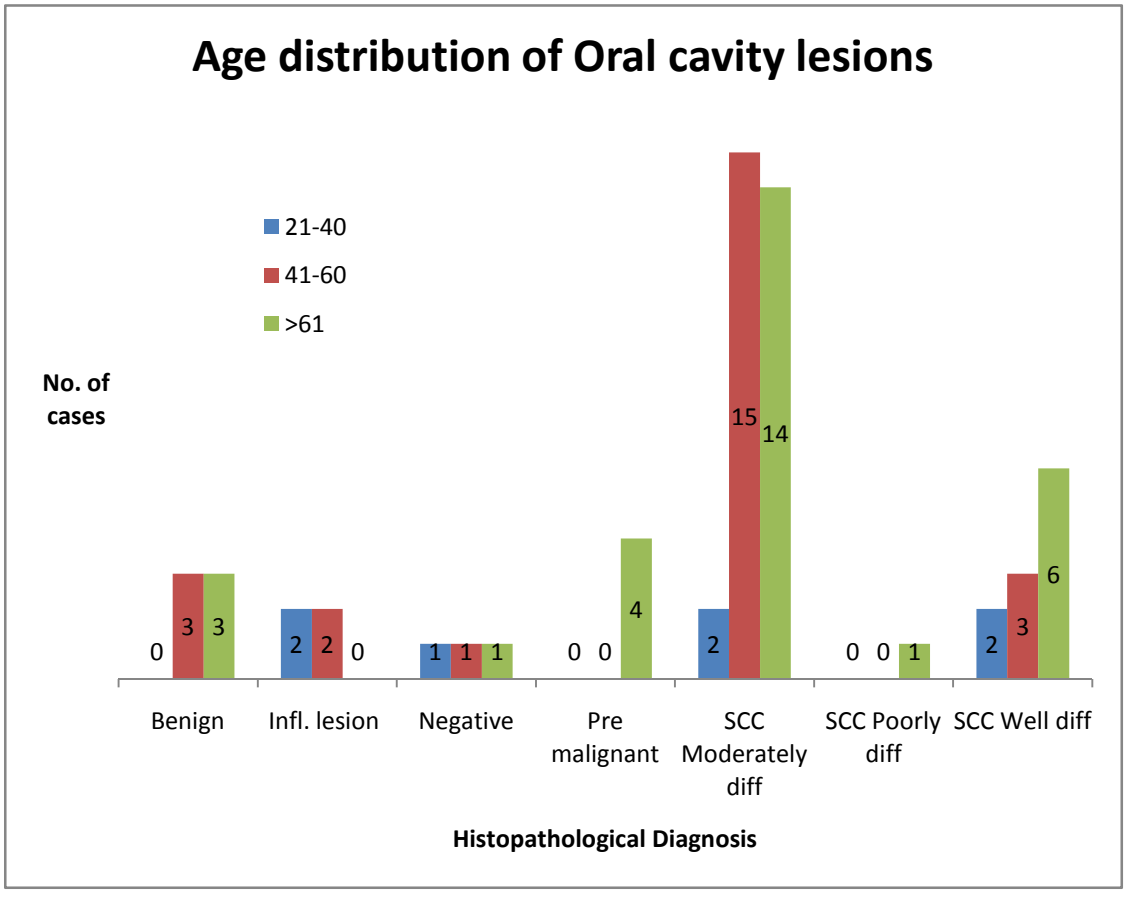


Similar to the smoking habit distribution, tobacco chewing habit distribution is also high among the older adults within the study group

Table - 5. **Age distribution of Oral cavity lesions**

Histopathological Diagnosis	Age distribution			Total
	21-40	41-60	>61	
Benign		3	3	6
Infl. lesion	2	2		4
Negative	1	1	1	3
Pre malignant			4	4
SCC Moderately diff	2	15	14	31
SCC Poorly diff			1	1
SCC Well diff	2	3	6	11
Grand Total	7	24	29	60

Chart - 5.

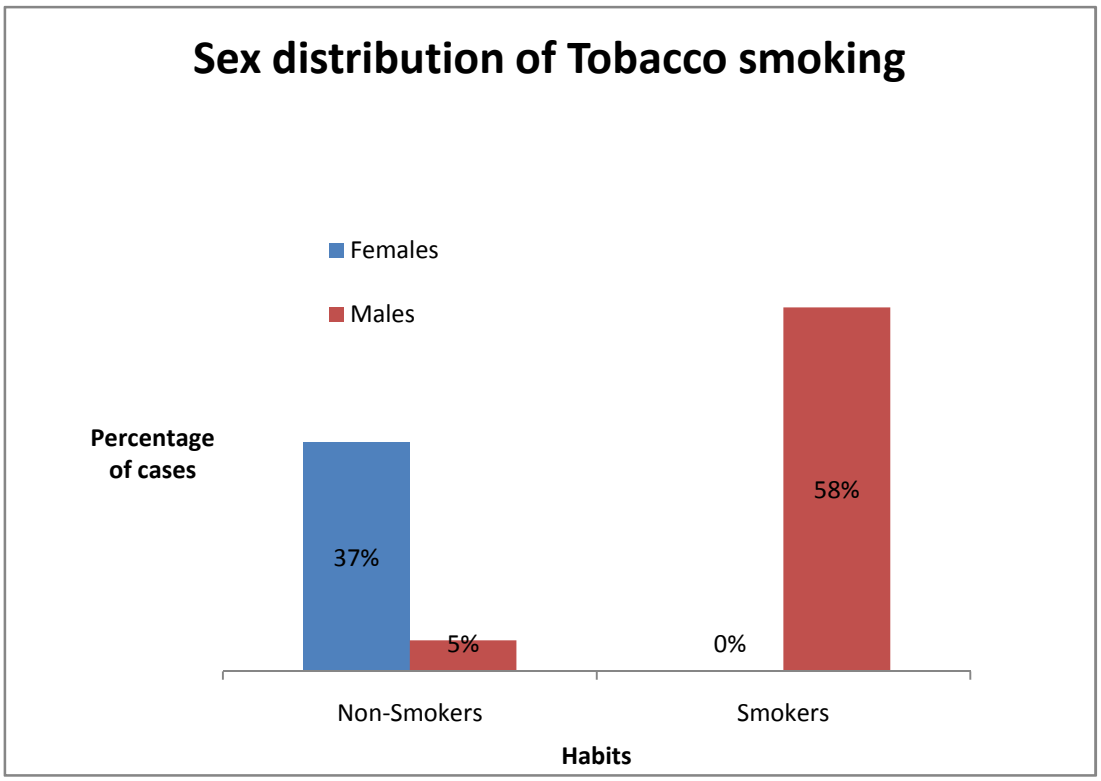


Squamous cell carcinoma is more prevalant among patients in the age group of 40 years and above.

Table - 6. **Sex distribution of smoking habit**

Smoking habits	Females	Males	Total
Non-Smokers	22	3	25
Smokers		35	35
Total	22	38	60

Chart - 6.

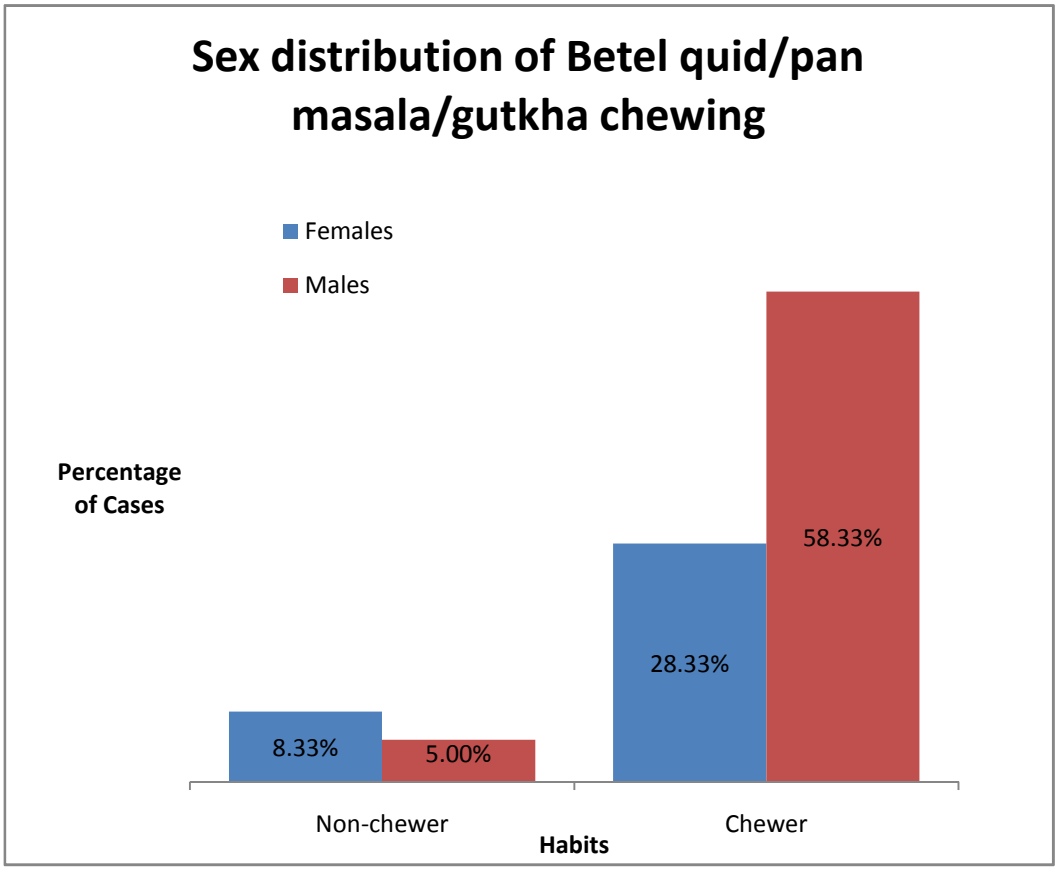


Smoking habit is absent among the females of this study group.

Table - 7. **Sex distribution of tobacco chewing habit**

Tobacco chewing habit	Females	Males	Total
Non-chewer	5	3	8
Chewer	17	35	52
Total	22	38	60

Chart - 7.



Tobacco chewing habit is seen among the females and it is around 28%. Among the both smoking and tobacco chewing distribution only 5% of males have no history of bad habits.

Table –8. **Sex distribution of p53 staining.**

Intensity of p53 staining	Females	Males	Total
-	7	5	12
+	2	3	5
++	7	13	20
+++	6	17	23
Total	22	38	60

Chart–8.

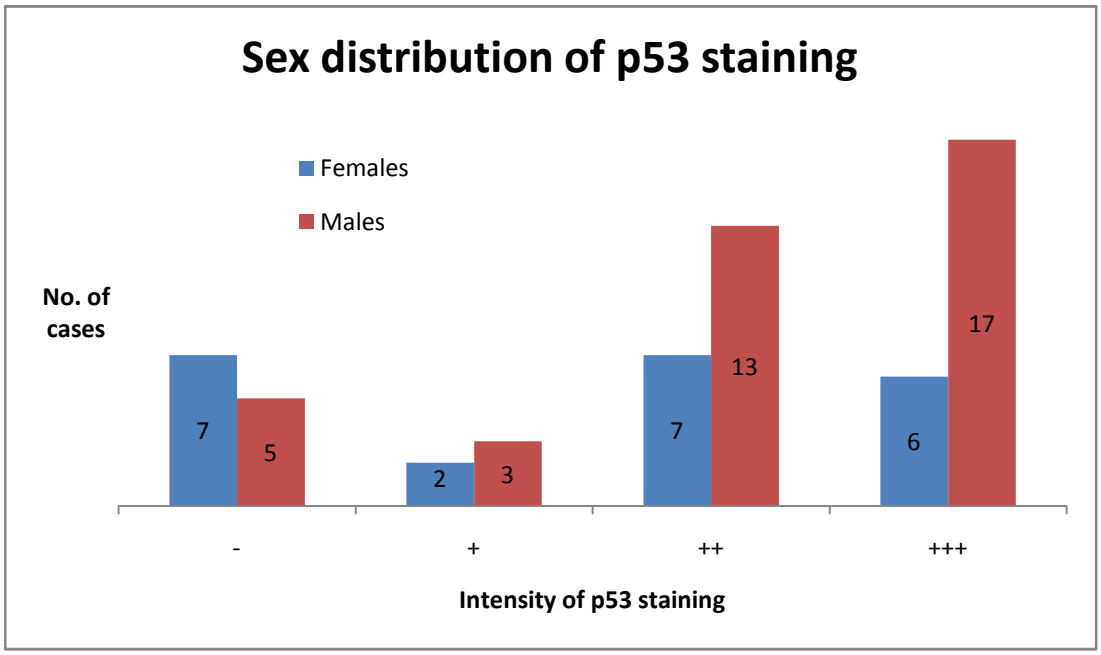
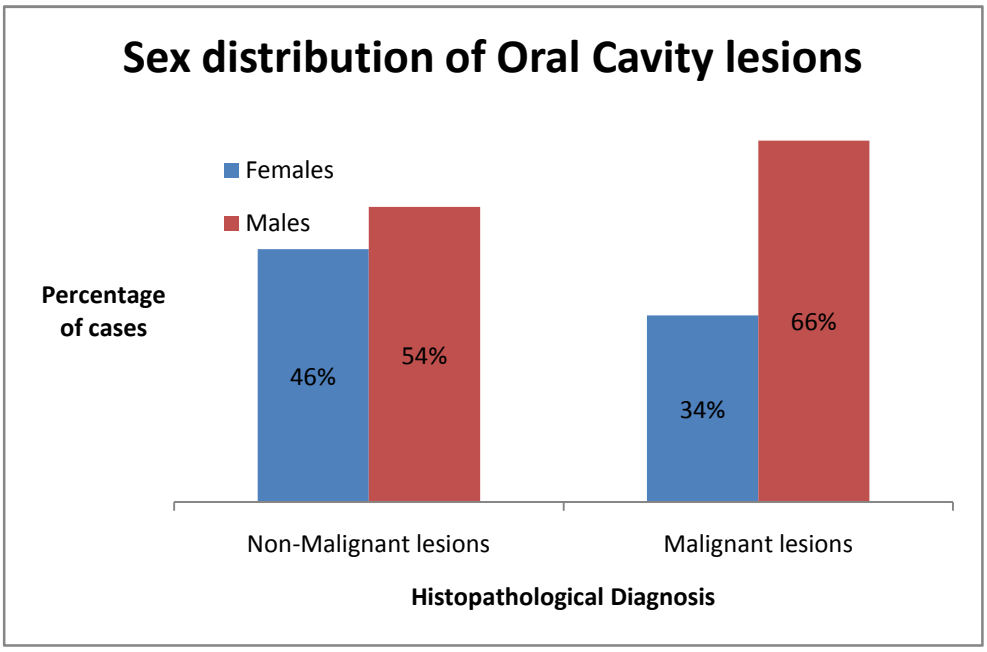


Table - 9. **Sex distribution of Oral Cavity lesions**

Histopathological diagnosis	No. of Cases		Total
	Females	Males	
Non-Malignant lesions	6	7	13
Malignant lesions	16	31	47
Total	22	38	60

Chart - 9.

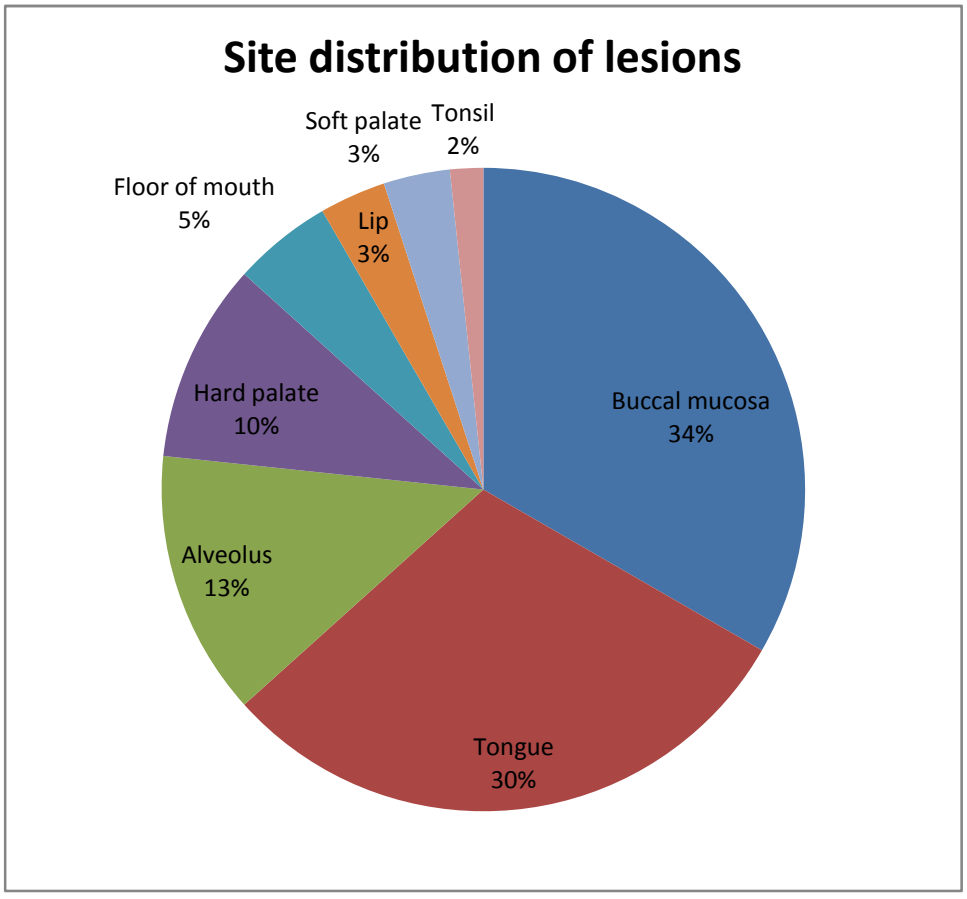


Non-malignant lesions show almost equal distribution among both sex whereas among malignant lesions there is increased prevalance among males.

Table - 10. **Site distribution of Oral cavity lesions**

SITE OF LESION	NO. OF CASES
Buccal mucosa	20
Tongue	18
Alveolus	8
Hard palate	6
Floor of mouth	3
Lip	2
Soft palate	2
Tonsil	1
Total	60

Chart - 10.

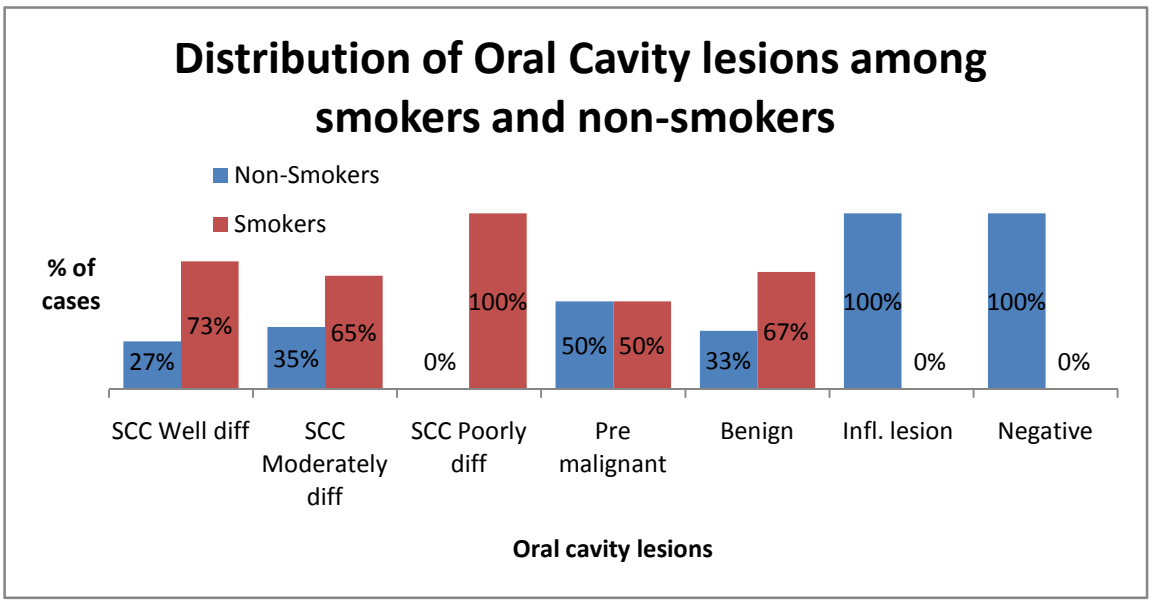


Buccal mucosa and tongue are the common sites involved in study group.

Table - 11. **Distribution of Oral Cavity lesions among smokers and non-smokers.**

Histopathological Diagnosis	Non-Smokers	Smokers	Total
SCC Well differentiated	3	8	11
SCC Moderately differentiated	11	20	31
SCC Poorly differentiated		1	1
Pre malignant	2	2	4
Benign	2	4	6
Inflammatory lesion	4		4
Negative	3		3
Total	25	35	60

Chart – 11.

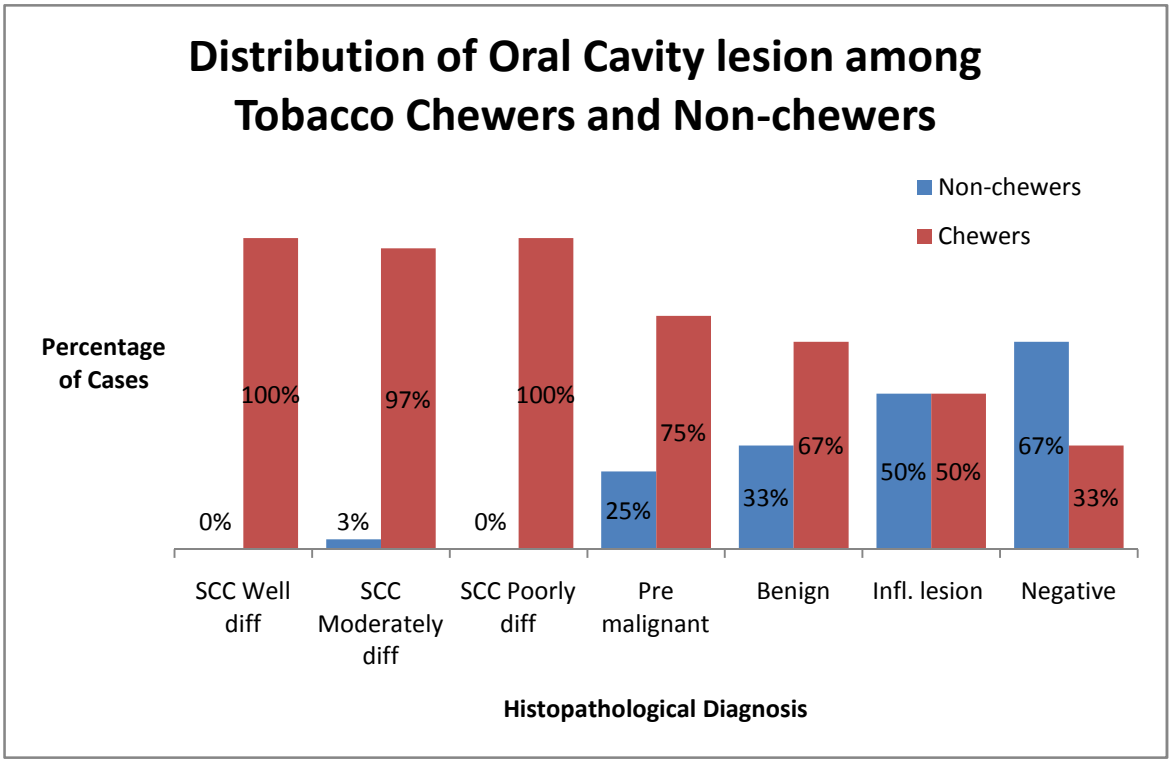


Percentage distribution of cases within each diagnosed cases shows that there is increased percentage of cases among smokers indicating that there is positive correlation between smoking and malignancy.

Table - 12. **Distribution of Oral Cavity lesions among Tobacco Chewers and Non-chewers**

Histopathological Diagnosis	Non-chewers	Chewers	Total
SCC Well differentiated		11	11
SCC Moderately differentiated	1	30	31
SCC Poorly differentiated		1	1
Pre malignant	1	3	4
Benign	2	4	6
Inflammatory lesion	2	2	4
Negative	2	1	3
Total	8	52	60

Chart- 12.

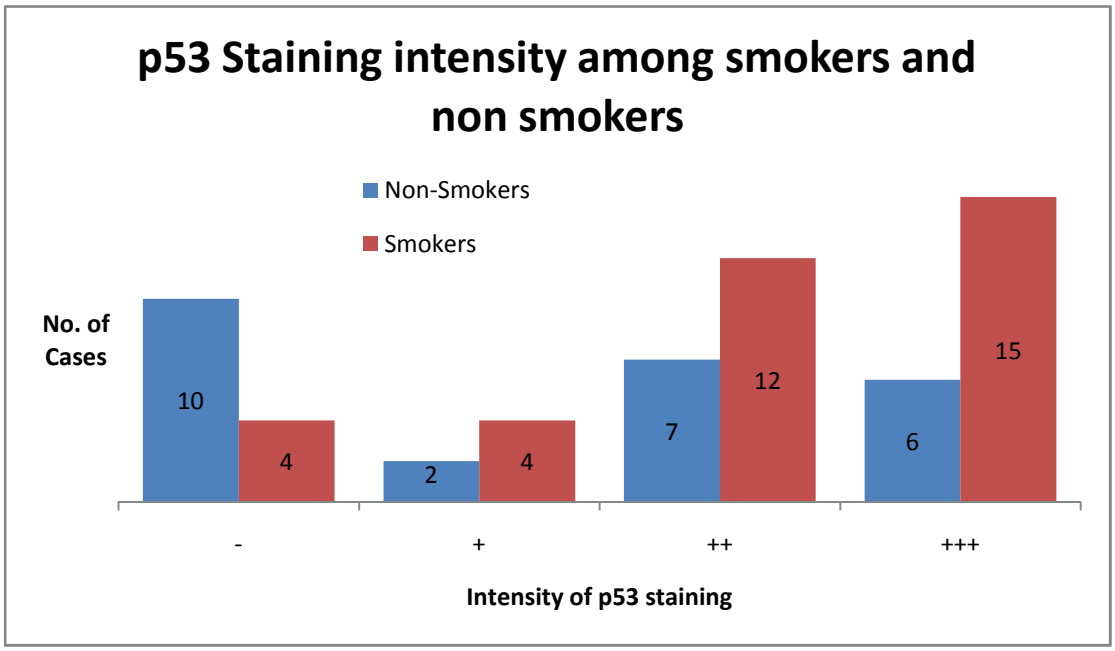


The data analysis between oral cavity lesions and the habit of tobacco chewing (Betel quid/Pan masala/Gutkha) shows positive correlation with malignant lesions and non-malignant lesions.

Table - 13. **p53 Staining intensity among smokers and non-smokers**

p53 Staining intensity	Non-Smokers	Smokers	Total
-	10	4	14
+	2	4	6
++	7	12	19
+++	6	15	21
Total	25	35	60

Chart - 13.

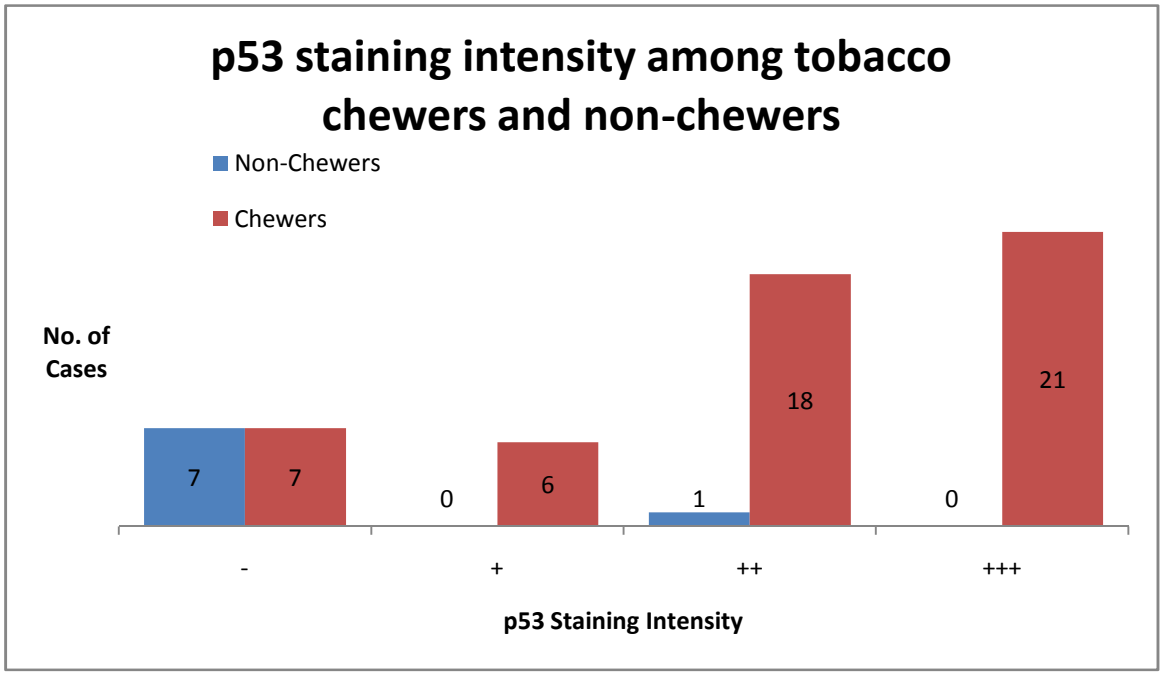


Data analysis of intensity of p53 staining among smokers and non-smokers shows positive correlation. Among non-smokers most cases show negative or weak positivity and among the smokers it shows strong positivity.

Table - 14. **p53 staining intensity among tobacco chewers and non-chewers**

p53 Staining Intensity	Non-Chewers	Chewers	Total
-	7	7	14
+		6	6
++	1	18	19
+++		21	21
Total	8	52	60

Chart- 14.

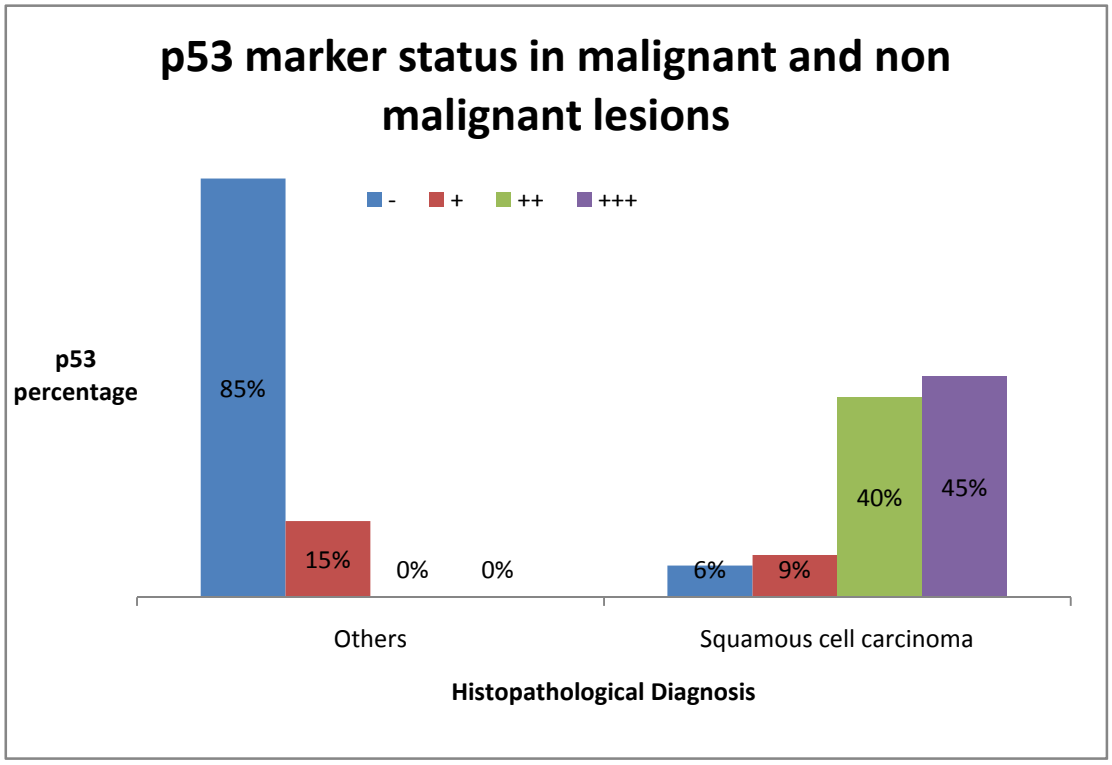


p53 staining intensity is strongly positive among the tobacco chewers and predominantly negative in patients with no history of tobacco chewing in any form.

Table - 15. **p53 marker status in Squamous cell carcinoma and other lesions.**

Histopathological Diagnosis	p53 Negative	p53 Positive	Total
Squamous cell Carcinoma	2	41	43
Non-malignant	12	5	17
Total	14	46	60

Chart - 15.



On percentage analysis of p53 immunostaining shows that positive staining is more among the cases reported as Squamous cell carcinoma and shows weak positivity or negative staining in non-malignant lesions.

Table -16. **p53 staining intensity in squamous cell carcinoma**

Squamous cell Carcinoma	p53 Staining intensity		Total
	Negative / weakly positive	Strongly positive	
SCC Well differentiated	2	9	11
SCC Moderately differentiated	3	28	31
SCC Poorly differentiated		1	1
Grand Total	5	38	43

Chart - 16.

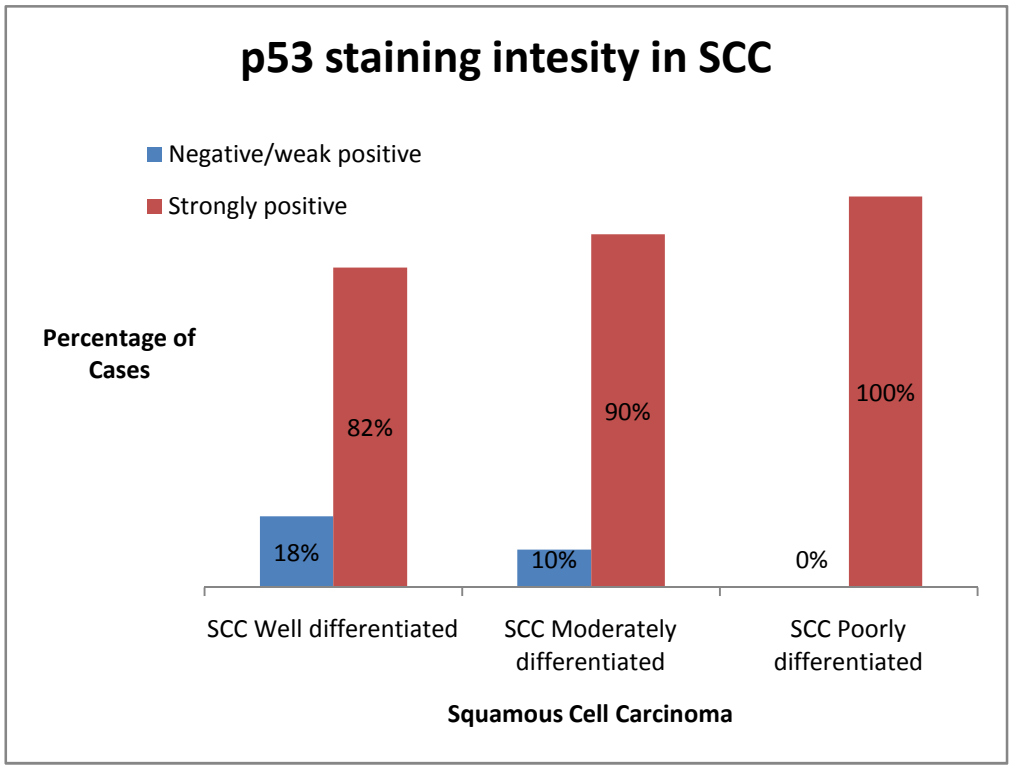
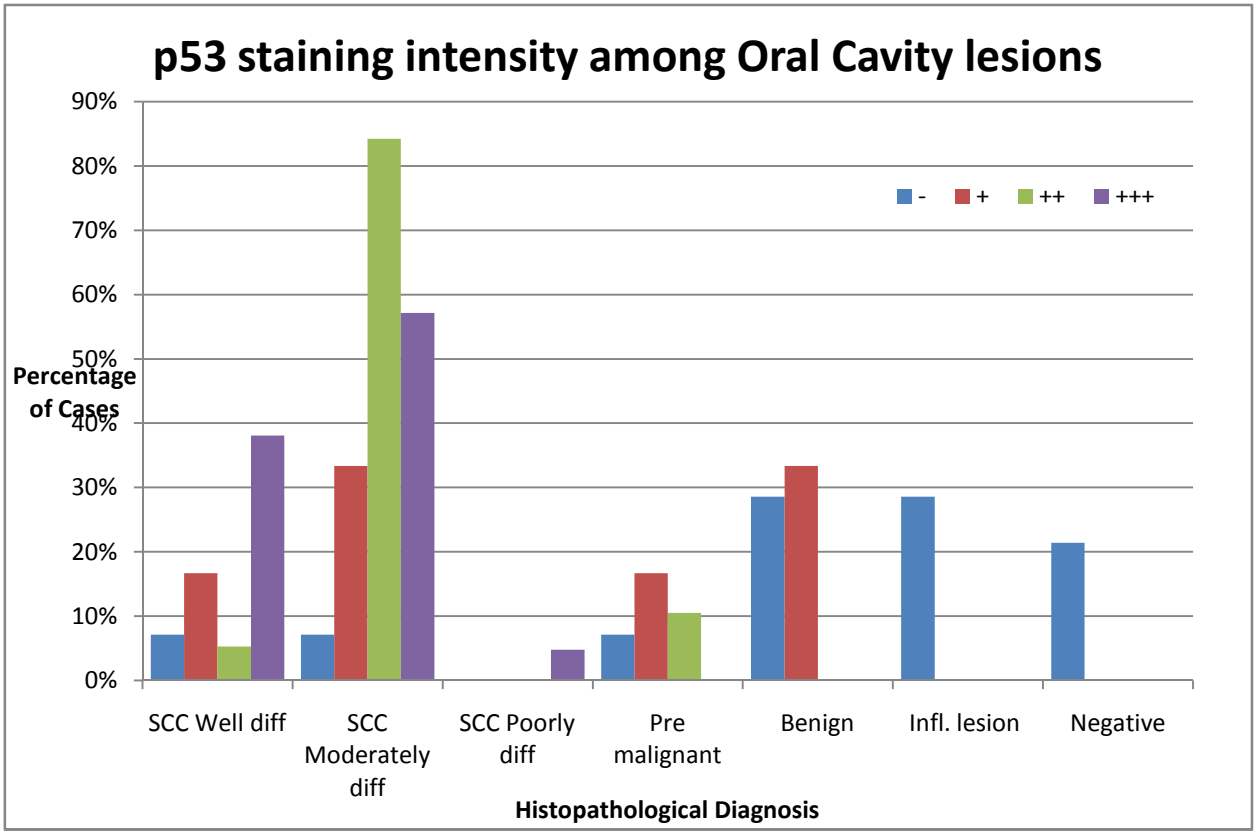


Table - 17. **p53 staining intensity among Oral Cavity lesions**

Histopathological diagnosis	p53 Staining intensity				Total
	-	+	++	+++	
SCC Well differentiated	1	1	1	8	11
SCC Moderately differentiated	1	2	16	12	31
SCC Poorly differentiated				1	1
Pre malignant	1	1	2		4
Benign	4	2			6
Inflammatory lesion	4				4
Negative	3				3
Grand Total	14	6	19	21	60

Chart - 17.



On percentage analysis of p53 immunostaining shows that positive staining is more among the cases reported as Squamous cell carcinoma and shows weak positivity or negative staining in non-malignant lesions.

In this study, over-expression of p53 oncoprotein was detected in 46/60 cases of lesions of oral cavity. The immunostaining reactions for oncoprotein p53 within the tumour cell populations were confined to the cell nuclei. Well differentiated tumours showed a strong immunostaining for p53 along the periphery of the tumour nests where the cells were actively proliferating, whereas the centre of the keratin pearls remained negative for the staining. Among the 46 cases of positive lesions, 21 cases showed strong staining (+++), 19 cases showed moderate staining (++), and 6 showed a weak staining (+). Verrucous carcinoma samples showed weak-to-strong staining. Normal epithelium in adjacent areas of the carcinomas remained negative for immunostaining (figures 2 – 9). The present study found no significant relationship in the expression of the oncoprotein with respect to the site of lesion. In most of the p53 positive cases, the subjects had a history of chronic chewing, both chewing and smoking. In the case of chewers, the staining intensity showed a significant correlation between the numbers of the betel quids chewed per day. Over-expression of the p53 oncoprotein showed a slight predilection for the male sex and most of them had smoking habits coupled with chewing [33/38 (87%) males vs. 15/22 females, (68%)].

Statistical analysis of p53 expression and Squamous cell carcinoma

To analyse the data, the Fisher Exact test of significance for association between two binary variables was used. It is useful replacement for the common X2 test in the case of 2x2 tables where some of the cells have very small - frequencies.

Table –18

p53	**Squamous cell carcinoma**	**Non-malignant**	**Total**
Positive	40	1	41
Negative	3	16	19
Total	43	17	60

The Fischer exact test statistic value is 0, which is significant at p value < 0.05. The test has a positive predictive value of 97% and negative predictive value of 84%, indicating a highly significant association between malignancy and p53 oncoprotein expression. The sensitivity of the test is 93% and specificity is 94%.

Statistical analysis of smoking and p53 expression

Table –19

p53	Smoking Habit - cigarettes/beedis per day		Total
	Less than 5	More than 5	
Negative	18	1	19
Positive	18	23	41
Grand Total	36	24	60

The Fischer exact test statistic value is 0.000151. The result is significant at $p < 0.05$, indicating a highly significant association between smoking cigarettes and development p53 positive staining in the cells. Hence a patient who smokes more than 5 cigarettes or beedis per day has a higher probability of developing p53 mutation than a patient with no history of smoking.

Statistical analysis of Betel quid/Pan masala/Gutkha chewing and p53 expression

Table – 20

p53	**Chewing Habit - sachets of Betel quid/Pan masala/Gutkha per day**		**Total**
	Less than 5	**More than 5**	
Negative	18	1	19
Positive	11	30	41
Grand Total	29	31	60

The Fischer exact test statistic value is 1E-06. The result is significant at $p < 0.05$, indicating a significant association between chewing tobacco products and developing p53 positive immunoexpression in oral lesions. Hence a patient who chews more than 5 sachets of betel quid/pan masala/gutkha is more likely to develop p53 mutation than a patient who does not have tobacco chewing habit.

DISCUSSION

DISCUSSION

A number of studies have shown that normal p53 acts as a molecular policeman which monitors genetic damage, or strand breaks in the DNA (Lane and Benchimol, 1990; Montenarh, 1992). It is postulated that p53 could have both a role in regulating transcription of genes that suppress cell proliferation, perhaps those affecting passage from late GI to S phase of the cell cycle (50), and a biological function as a GI checkpoint control allowing the repair of DNA damage (48). The wild-type of the protein has a shorter half-life when compared to mutant variety of the protein which is more stable and is, in part, responsible for malignant transformation (67). Over-expression of the p53 oncoprotein has been reported in oral squamous cell carcinomas. The staining intensity of p53 also shows a linear correlation with increasing degrees of dysplasia and the appearance of SCC (65). Since more than 70% of the cases that have been studied here show the presence of p53 oncoprotein, it is possible that mutation of p53 plays a role in the progression of oral malignancies from a state of dysplasia to squamous cell carcinomas. It is also possible that there may be mutation in - one of the alleles, and the remaining allele is lost during a later period. It has been suggested that mutation in one of the alleles and/or loss of the remaining allele leads to malignant transformation (21). The fact that the expression of the protein is higher for oral SCCs demonstrating more than 40% of cells with binding to proliferating cell nuclear antigen (PCNA) shows that malignancy might be promoted due to an increase in the cell cycle somehow related to p53 oncoprotein expression (76).In a study by Soussi et al. (1994) have suggested that more than 90% of missense

mutations account for the immunohistochemically detectable p53 whereas nonsense mutations, which arise from alterations in nucleotide sequences that convert triplet codons for given amino acids into termination codons, and/or deletions which are not immunohistochemically detectable. The loss of function of the wild-type allele and/or the presence of missense mutations may, in part, explain the increased positive p53 staining in oral carcinomas. To analyse this present study used immunohistochemical technique to retrieve the antigen. Further experimental evidence is needed to support this view. As the staining reaction of the protein is limited to the nucleus, it appears that the mutant protein that have acquired a dominant transforming activity remain in the nucleus (77). The process of carcinogenesis is presumed to be taking place in a step-wise manner and multiple genetic changes are required before a normal cell turns fully neoplastic (78). Etiological factors such as tobacco and alcohol have been shown to induce a variety of genetic changes in human neoplasia including head and neck, and oral squamous cell carcinomas (79). Tobacco has also been implicated in the causation of lung cancer (80) and bladder cancer (81).

Several investigators have suggested that unrepaired DNA damages might lead to mutation in the p53 gene by inducing G+T transversions (82). The carcinogens like Tobacco specific nitrosamines (TSNA) and areca nut specific nitrosamines (ASNA) are able to induce mutations in exons 5-9 of p53 gene and the most commonly observed changes are the G→A transitions (83) and G→T transversions (84). It has also been suggested that in vitro immortalization is a rate-limiting step in carcinogenesis. Recent studies have shown that p53 is needed for immortalization and that the gene may get inactivated by factors such as HPV types 16 and 18 (85) or it

may have some role in programmed cell death or apoptosis (63). The wild-type p53 gene product can reduce the transforming efficiency of many oncogenes and can suppress tumour growth, whereas mutant forms can lead to malignant transformation, especially when the protein is co-expressed with cellular oncogenes such as ras (86).

The results of this study although differ from that of Ranasinghe and his associates (1993) stating that p53 expression is low in oral squamous cell carcinoma, strongly suggests that p53 oncoprotein is involved in the tumourigenesis of oral SCCs. Recent studies have shown that p53 may play an important role in the causation of oral cancers in habitual tobacco betel quid chewers, and that the expression of p53 oncoprotein shows a relationship with the number of quids per day and the frequency of chewing (87). Studies have also shown that ras oncogene and p53 gene form the part of the machinery which controls cellular proliferation and or differentiation. In normal cells, cyclin-dependent kinases (CDK) exist in multiple active forms containing cyclins, proliferating nuclear antigen (PCNA), and p21 (88). As such, p21 is considered to be an inhibitor of cellular proliferation(94). Studies by Xiong, Zhang and Beach (1993) have shown that there is loss of p21 protein from CDK complexes in cells obtained from patients with Li-Fraumeni Syndrome (LFS), a syndrome that represents an inherited familial disorder with a relatively high susceptibility for early onset of various tumours (89). It is known that LFS patients already have a copy of p53 mutant allele while the remaining wild-type p53 allele might undergo mutation (90) and that their cells carry no known DNA viruses (91). Therefore, the loss of p53 gene may alter normal functioning of the cell cycle and the functions of other cell cycle-associated proteins which act to cooperate during malignant transformation (49).

It is possible that the normal tumour suppressor function of p53 is mediated through p21 or p21 is dependent upon the p53 pathway to effect the changes in cell proliferation and CDK complexes (92). Although the incidence of ras mutations is comparatively low in oral squamous cell carcinomas in Western countries (93), H-ras analysis in the Indian oral cancer patient group (Bombay) has revealed a high percentage of tumour-associated loss of heterozygosity (LOH) in about 35% of the cases (72). A still higher percent of ras mutation (50-70%) has been documented in oral SCC from South India and this study has also shown that the ras oncogene is, in part, responsible for malignant transformation (94). Hence, it is possible that there may be co-expression of p53 with the mutation of H-ras and that these mutated proteins are essential for malignant transformation or may have a synergistic effect on malignant transformation at least in some oral cancers caused by tobacco betel quid chewing. Also, the ingredients of the betel quid may play a decisive role in the causation of p53 mutations. As the betel quid in Tamilnadu does not contain any additional spices (95) as opposed to Northern India, it may be possible that this patient group lack the protective effects, if at all they have any, from these added condiments.

The phenolic compounds in areca nut may trap nitrites and may exhibit a strong anticlastogenic and antimutagenic effect (96), but these compounds may show chromosome damaging effects when used in combination with other ingredients of the betel quid such as tobacco, catechu, betel leaf, lime, and spices and flavouring agents (97). Studies have shown that the exposure time to the carcinogens in tobacco and the age at the very first exposure is quite significant in the causation of oral cancers (97). Such factors like the duration of exposure to tobacco, the amount of tobacco, the age

at the very first exposure are also important as the areca nut-related phenolics stay in the saliva of the habitual chewers only for a limited time (98); it usually disappears soon after expectoration of the macerated betel quid mixture (99). There is epidemiological evidence that the tobacco and betel quid chewers of Tamilnadu usually keep the chewing mixture for prolonged periods in their mouth and most of them start the chewing habits at an early age (95). This may increase the exposure time to the carcinogens when compared to subjects from other places such as - Sri Lanka (10-30 min.), (100). Unlike other places, in Tamilnadu, the habitual chewers prefer slaked lime to stone lime (101) which obviously will increase the pH along with tobacco thereby inflicting more damage onto the tissues (102). Reactive oxygen species generated from the chewing mixture is known to cause genetic damage in a sequential dose-dependent manner (103) and cell death (104). But there is no conclusive evidence to show that the presence of excess lime and the lime-induced tissue injury would affect the p53 expression.

It appears that some carcinogens in (105) tobacco act only at certain sites of the gene (69) and that moderate to heavy smokers, unlike non-smokers, are likely to have multiple mutations (44). It is also likely that there exists a synergistic effect between the various etiologic factors and the incidence of genetic damage (106). It has been shown that nutritional deficiencies and tobacco chewing and smoking habits are more prevalent in the rural population of India, which constitutes more than 80% of the total population (107). Chilli, a main ingredient of the diet in the southern parts of India, is known to contain mutagenic and tumour promoting agents (108) have shown that there is an 11-13 fold increase in the incidence of oral cancer in habitual chewers who

do not prefer a vegetarian diet. There is epidemiological evidence that the relative risk for beedi is much higher when there are other habits such as tobacco chewing and alcohol drinking (109). The fact that most of the male subjects in the present study had chronic tobacco betel chewing and beedi smoking habits may account for the increase incidence of genetic damage which, in turn, predispose them to oral cancer. As reported by many investigators, alcohol may have a synergistic effect since most people in the rural areas in Southern India use home-brewed alcohol which contains about 40-50% ethanol (95). In effect, the aforesaid etiologic factors may act in a sequential manner to bring about the malignant changes.

The number of betel quids chewed per day, exposure to the carcinogens and the presence of more than one habit is important in the process of carcinogenesis. 52/60 (87%) of the patients from the present study were chewers. Above normal levels of natural radiation, the carcinogens in the tobacco betel quid mixture, and the beedi smoke alone or in combination with one another may play a role in the incidence of mutation outside the 'conserved regions' of p53. Further studies using advanced techniques such as polymerase chain reaction (PCR) coupled with direct DNA sequencing may help elucidate the molecular aspects of oral cancers in tobacco and betel quid chewers.

It has been suggested that over-expression of tumour suppressor genes is likely to be associated with dysfunction of a common regulatory pathway in the case of proliferation and differentiation (110). Many researchers have shown that keratinocytes transfected with HPV types 16 and 18 tend to undergo malignant transformation on exposure to tobacco-related carcinogens alone or in combination

with tumour promoters such as N-nitroso-N-methylurea (NMU) and phorbol esters (111). It is possible that the tumour suppressor gene p53 may be inactivated by the papilloma virus and that E6 protein of the virus can bind to and promote degradation (112). As the gene acts to control transcription, its inactivation may predispose the affected tissues to malignant transformation. Cases have been reported where there is a negative relationship between the suppressor gene inactivation and the presence of HPV (113). Virus-induced changes along with the tobacco-related carcinogen-induced damage may sequentially make oral keratinocytes more prone to malignant transformation (114).

The ingredients of the betel quid also play a major role. Unlike other places, in Tamilnadu, all the substances are used fresh and most patients have a tendency to leave the chewing material in their mouth for longer periods, and sometimes even during sleep (115). This may, in turn, increase the time of exposure to the carcinogens (95). Lime is known to contain radioactive substances which may emit α-particles as high as 0.35 pCi which is equivalent to the amount of alpha-particles emitted from 0.53 mg/Kg u (116). Thus, it could be hazardous for people who chew continuously or who are exposed to the excess amounts of lime as it may cause damage to the tissues and the genetic material. Yet another possibility is that the hot and spicy food items, a habitual action in southern parts of India, may give rise to a variety of carcinogens. A detailed dietary analysis is required to establish the exact role of diet in oral, and head and neck cancers. Also, southern parts of India is one of the areas where there is relatively high inbreeding which may, in part, account for the increased rate of genetic susceptibility.

In summary, in this tertiary care hospital based on the analysis of results p53 plays a major role in OSCCs and this p53 overexpression may be caused by the factors such as tobacco chewing, betel quid chewing, pan masala or gutkha chewing habits. The genotoxic effects of tobacco, alcohol and diet-related factors may have a field cancerization effect on the mucosa of the upper aerodigestive tract and may thereby predispose the whole epithelium to malignant transformation (117). However, the results of work done by Kannan et al. (1993) taken together with the present study suggests that a correlation between the overexpression of p53 protein and ras mutation is very likely. Further analysis of these cases at the molecular level might give some insight into functional aspects of the etiological factors in the development of oral cancers in habitual tobacco/betel quid chewers.

CONCLUSION

CONCLUSION

The results of the study on the levels of p53 immunoexpression show that there is overexpression of p53 oncoprotein in SCCs of oral cavity at this tertiary care centre – Tirunelveli Medical College, Tirunelveli. The overexpression of p53 in oral cavity squamous cell carcinoma samples from Tirunelveli is strongly associated with the number of betel quids chewed and the number of cigarettes or beedis smoked per day. Other factors such as diet-related factors, natural radiation, relatively high genetic susceptibility, and/or the presence of transforming proteins of viruses may act sequentially to make the cells more prone to malignant transformation. p53 oncoprotein is likely to be involved in the promotion and/or progression of the squamous cell carcinomas, and the discovery of p53 mutations in human SCCs suggests that alterations in tumour suppressor genes play an important role in some oral cancers (118). Although the protein is implicated in cell proliferation and/or differentiation, more investigations are needed to decipher the molecular mechanisms involved.

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ANNEXURE

ஆராய்ச்சித் தகவல்தாள்

திருநெல்வேலி அரசு பொது மருத்துவமனைக்கு வாய்ப்புண் பாதிப்புகளுடன் வரும் நோயாளிகளுக்கு வாய்ப்புண் புற்றுநோய் உள்ளதா மற்றும் அதற்கு சிகரெட்/பீடி புகைப்பழக்கம், புகையிலை/பான்மசாலா /குட்கா உபயோகிக்கும் பழக்கத்திற்கும் எந்த அளவிற்கு காரணியாக உள்ளது என்பதை கண்டறிய நவீன பரிசோதனைப் பற்றிய ஆராய்ச்சி நடைபெற்றுவருகிறது .

வாய் புண்ணுடன் வரும் நோயாளிகளுக்கு வாய்ப்புண் புற்றுநோய் உள்ளதா மற்றும் அதற்கு /பீடி புகைப்பழக்கம், புகையிலை/பான்மசாலா /குட்கா உபயோகிக்கும் பழக்கத்திற்கும் எந்த அளவிற்கு காரணியாக உள்ள என்பதை கண்டறியும் நவீன பரிசோதனை எந்த அளவிற்கு பயனளிக்கும் என்பதை அறிந்துகொள்வதே இந்த ஆராய்ச்சியின் நோக்கமாகும்.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். முடிவுகளை அல்லது கருத்துக்களை வெளியிடும்போதோ அல்லது ஆராய்ச்சியின் போதோ தங்கள் பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம் .

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தைப் பொறுத்தது மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

இந்த சிறப்புப் பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போதோ அல்லது ஆராய்ச்சியின் முடிவிலோ தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம்   பங்கேற்பாளர் கையொப்பம்

தேதி:

இடம் :

ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சித் தலைப்பு:

பெயர்:

வயது :

பால் :

தேதி:

உள்நோயாளி எண்:

ஆராய்ச்சி மைய எண் :

இந்த ஆராய்ச்சியில் எனது அல்லது என் குழந்தை
தன்னார்வமாக கலந்து கொள்ள அழைக்கப்பட்டது.

இந்த ஆராய்ச்சியில் பங்கேற்பதற்கு முன் அதன் .

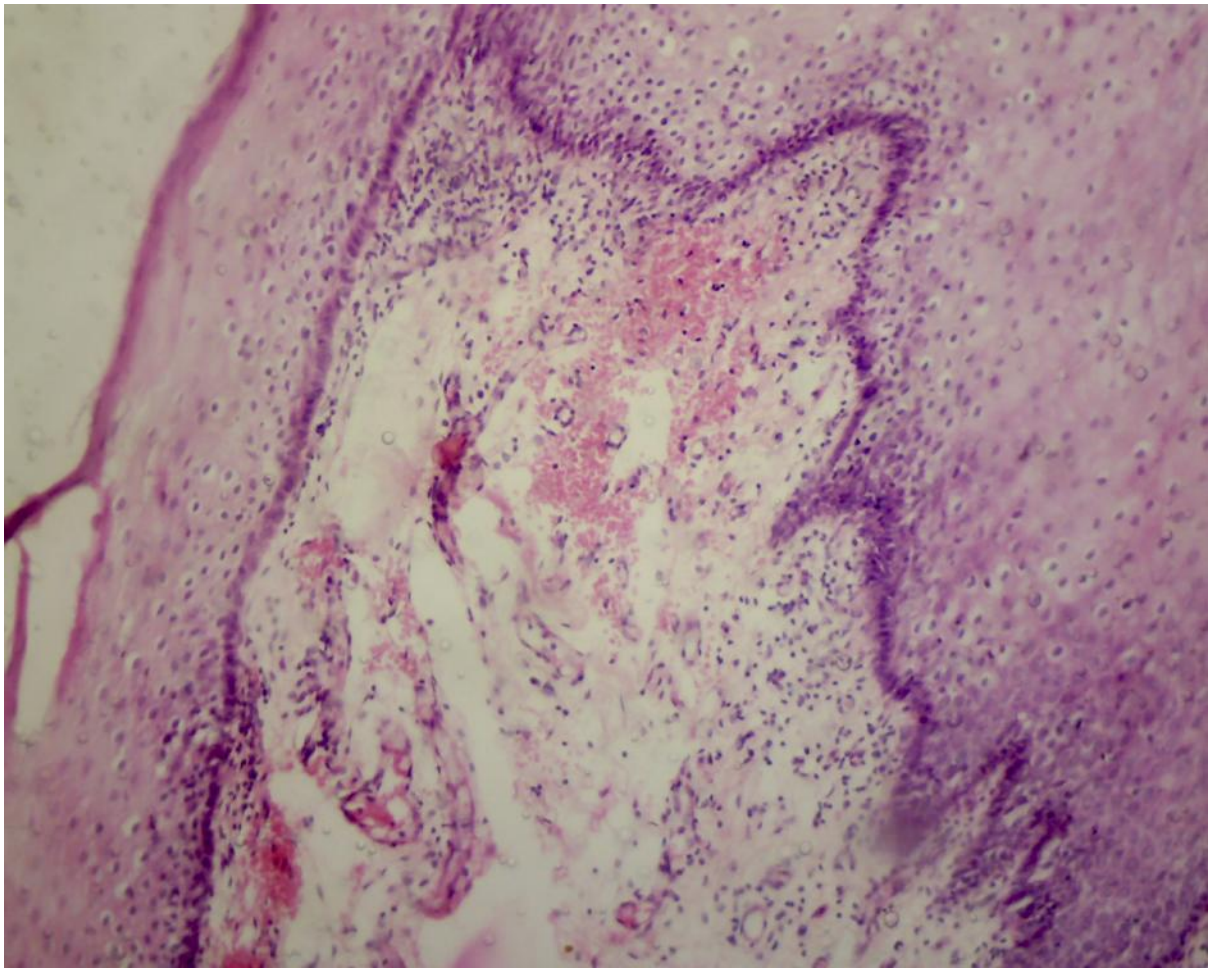

Figure – 2.Normal squamous epithelium with acanthosis.

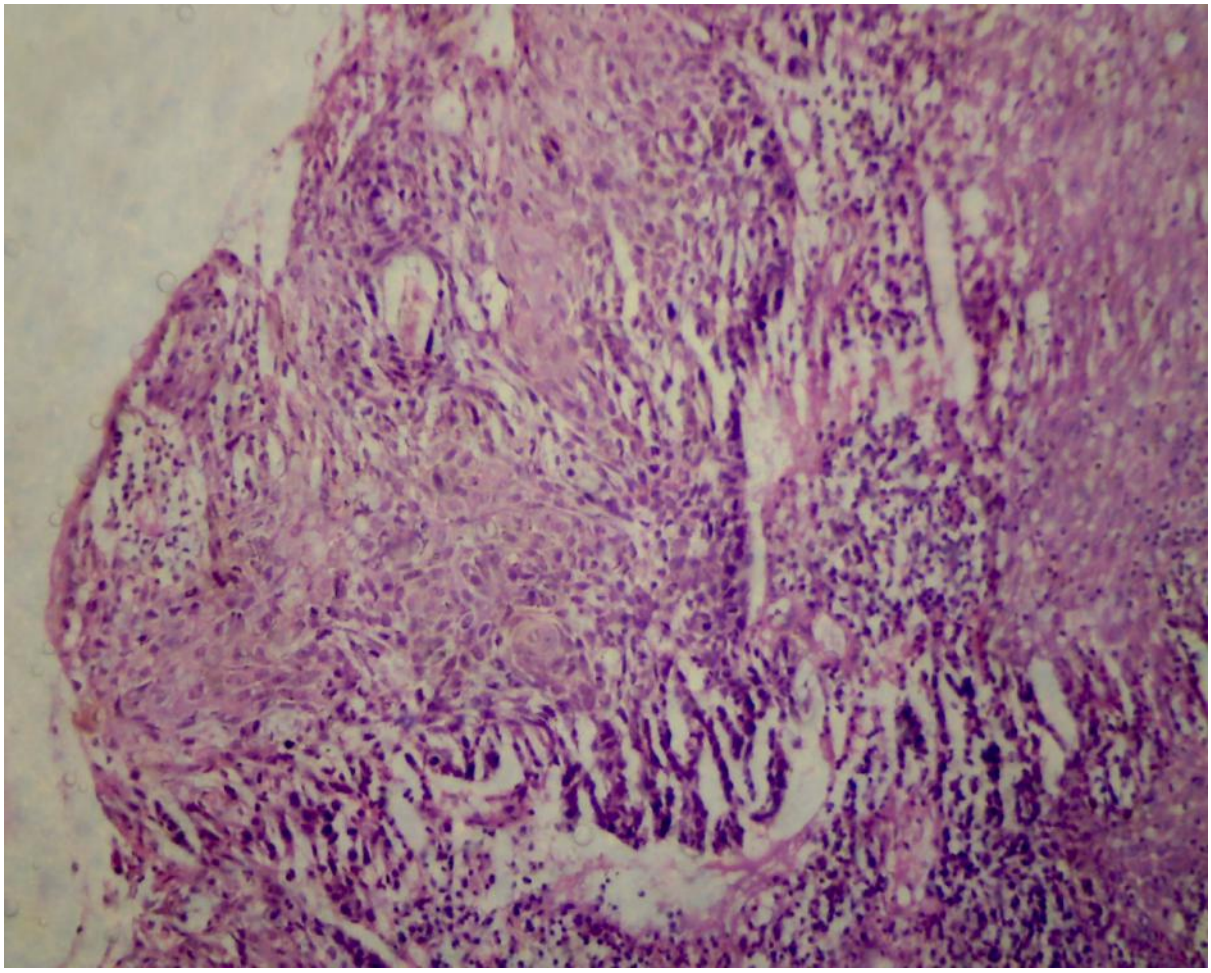

Figure – 3. Well differentiated squamous cell carcinoma

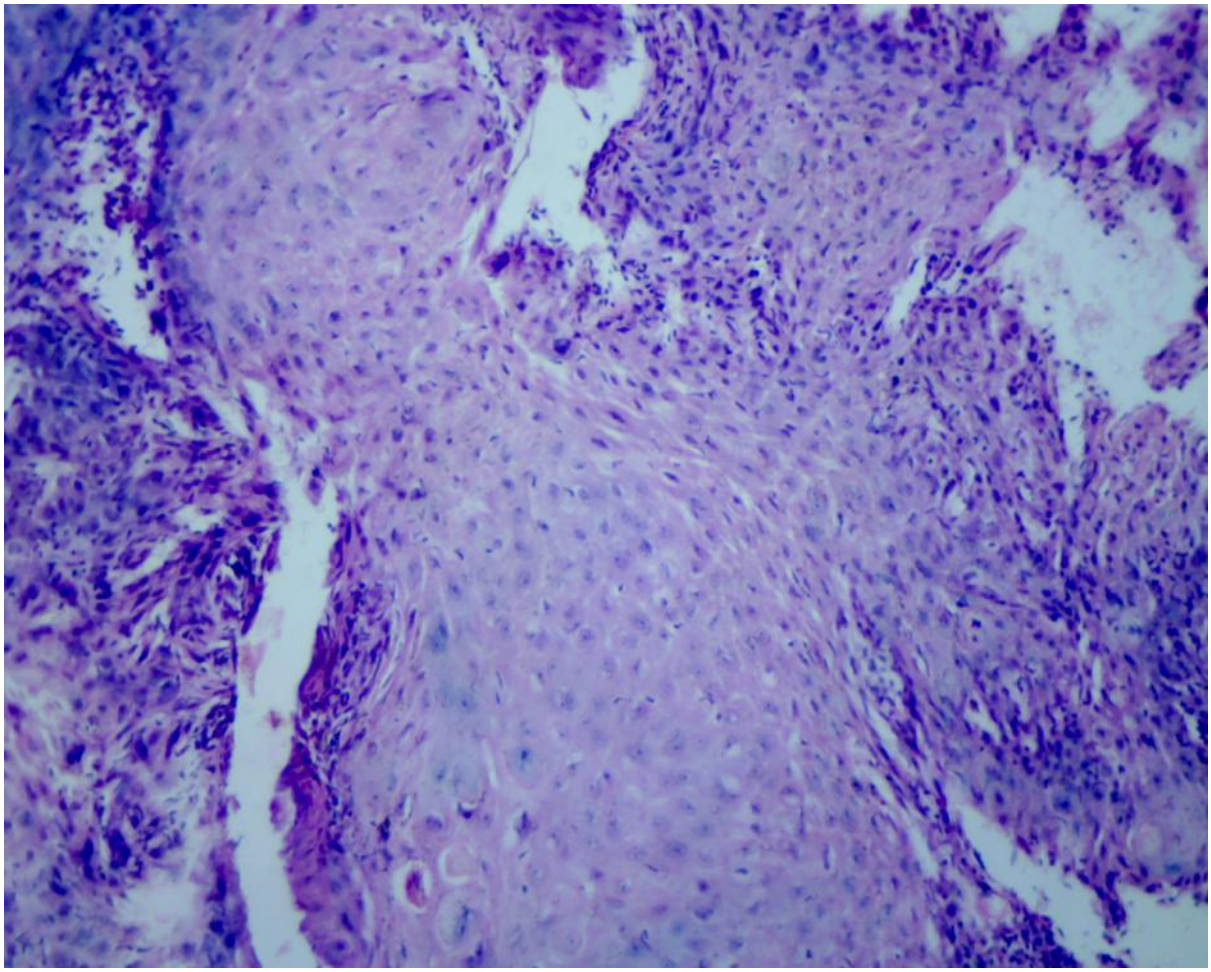

Figure – 4. Moderately differentiated squamous cell carcinoma

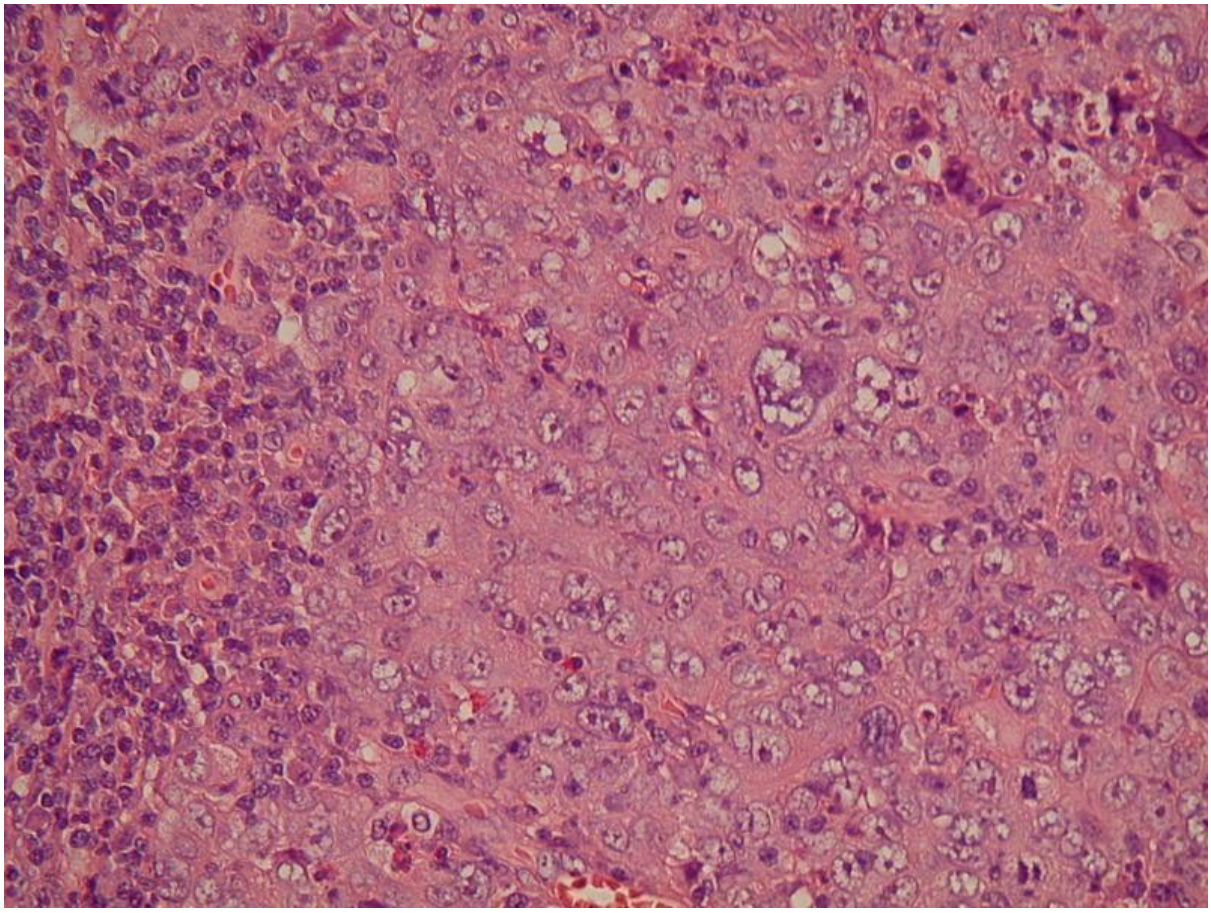

Figure – 5. Poorly differentiated squamous cell carcinoma

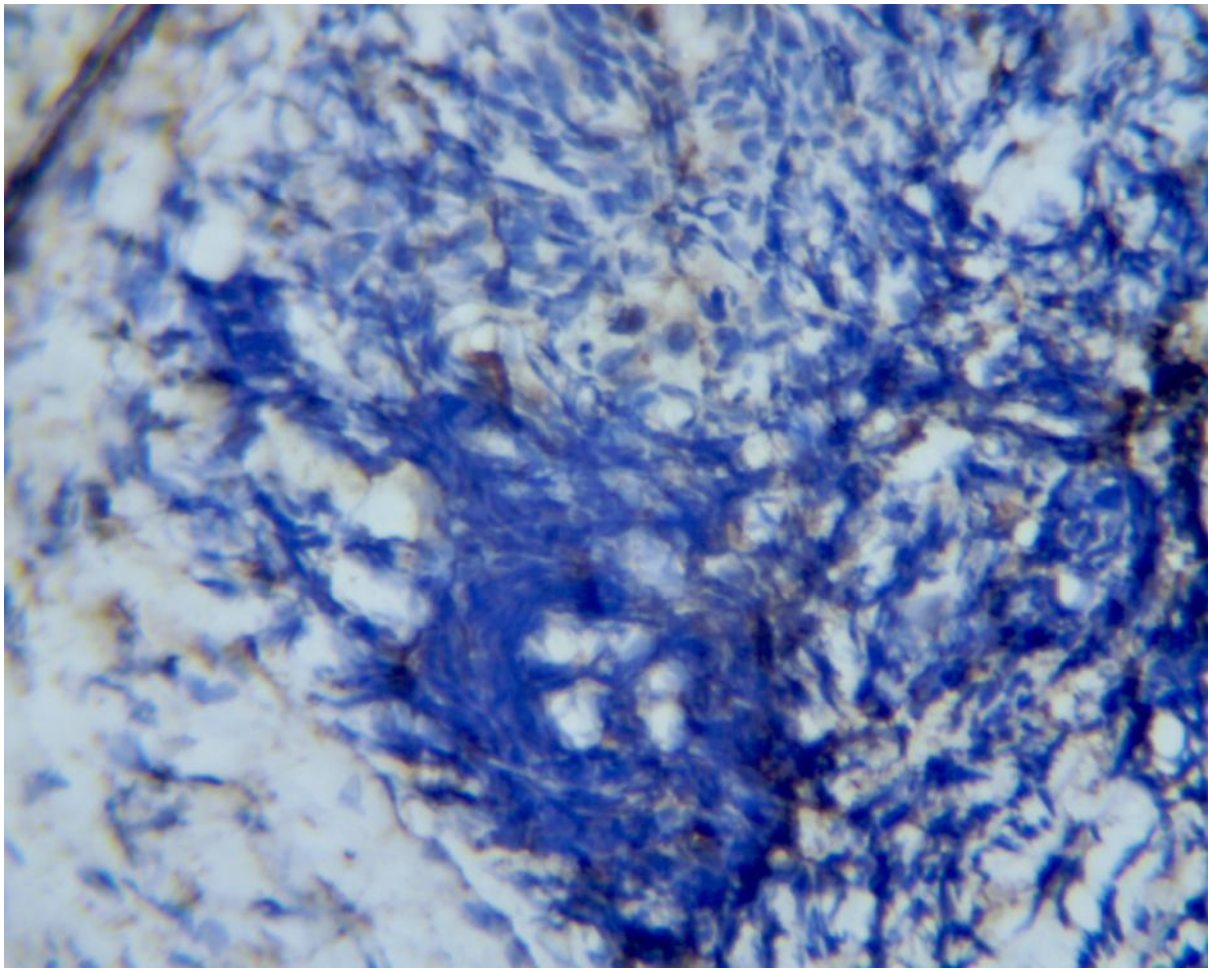

Figure – 6.Case showing negative staining for p53 marker

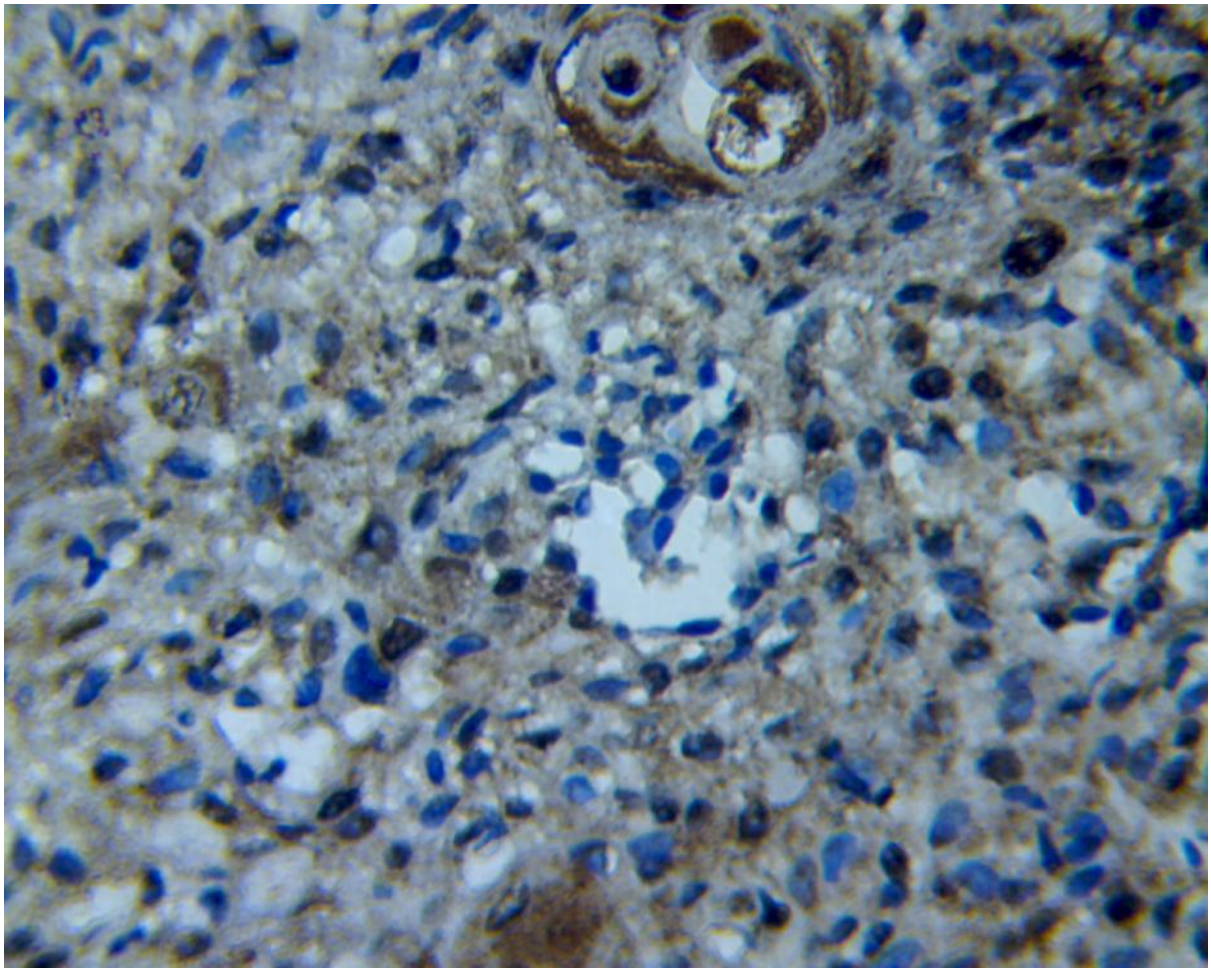

Figure – 7.Case of moderately differentiated squamous cell carcinoma showing 1+ staining for p53 marker.

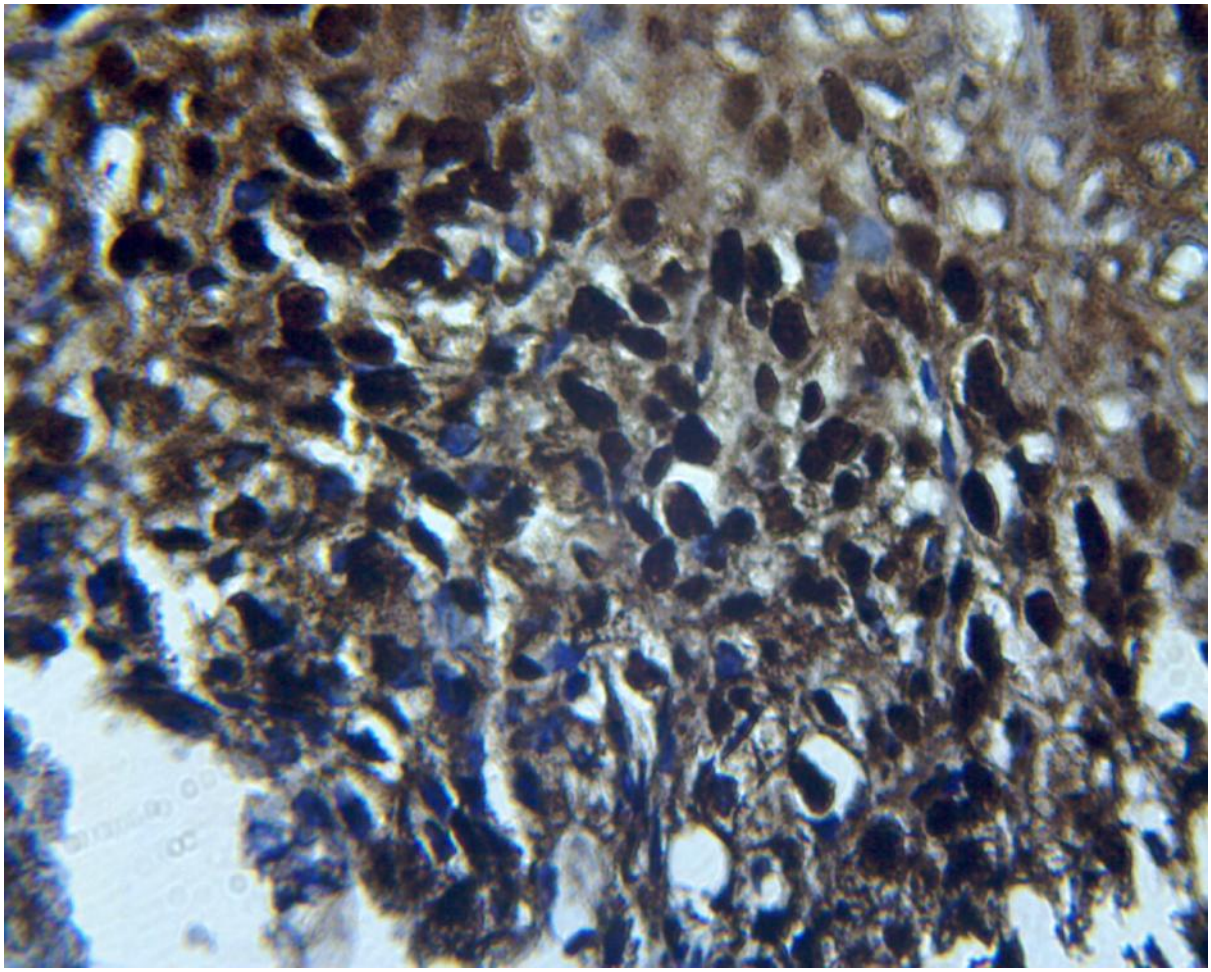

Figure – 8.Case of moderately differentiated squamous cell carcinoma showing 2+ staining for p53 marker.

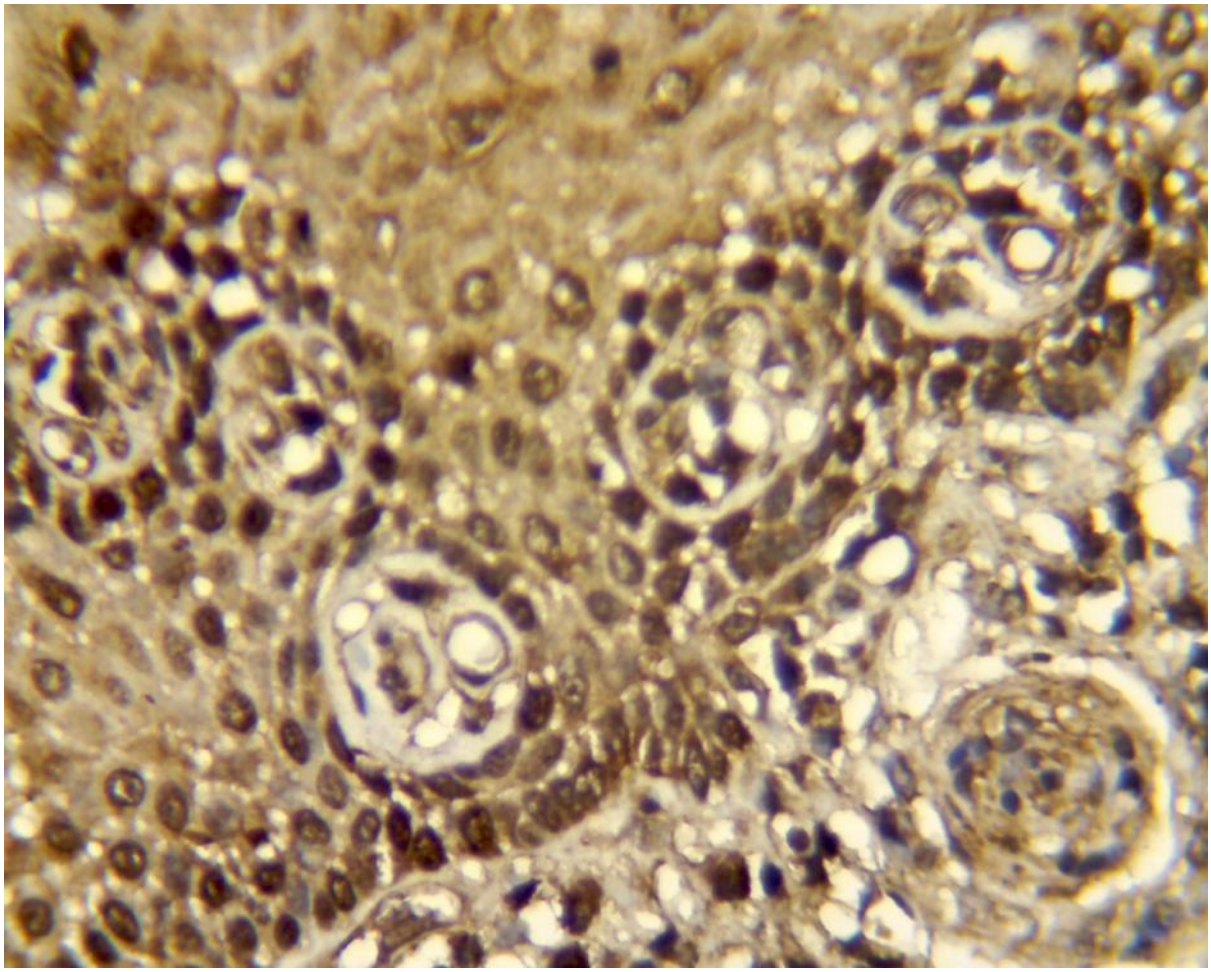

Figure – 9. Case of well differentiated squamous cell carcinoma showing 3+ staining for p53 marker

IMMUNOHISTOCHEMICAL ANALYSIS OF P53 EXPRESSION IN ORAL CAVITY LESIONS AND ITS RELATION WITH POOR PERSONAL HISTORY
MASTER CHART

S.NO	HPE NO	AGE/SEX	HISTOPATHOLOGICAL SPECIMEN	NO OF CIGARETTES/BIDIS SMOKED/DAY	NO. OF SACHETS OF PAN, GUTKHA OR BETEL QUID CHEWED/DAY	HISTOPATHOLOGICAL DIAGNOSIS	p53 STAINING INTENSITY
1	3455/14	83/M	?Ca cheek	12	10	Well diff scc	+++
2	3507/14	34/M	Growth right cheek	3	12	Well diff scc	-
3	3549/14	75/M	?Ca lower lip	12	10	Well diff scc	+++
4	3560/14	55/M	Growth ant 2/3 tongue	3	5	Suggestive of scc	-
5	3616/14	48/F	Growth floor of mouth	0	15	Invasive scc	+++
6	3711/14	77/M	Ca alveolus	12	5	Well diff scc	+++
7	3834/14	65/F	Ca cheek	0	12	Well diff scc	+++
8	3881/14	60/F	Ca alveolus	0	15	Keratinising scc	+++
9	20/15	46/F	Ca cheek	0	12	Invasive keratinising scc	+++
10	49/15	34/M	Ca tongue	15	3	Well diff scc	+
11	77/15	74/F	Growth tonsil	0	3	Mod diff scc	++
12	212/15	55/M	Ca tongue	4	10	Mod diff scc	++
13	233/15	50/M	Buccal alveolar growth	5	5	Ulcer with florid pseudoepitheliomatous hyperplasia with a foci of moderate dysplasia	-
14	311/15	79/M	Growth left buccal mucosa	20	12	Keratinising invasive scc	+++
15	362/15	35/F	Ca anterior 2/3rd of tongue	0	6	Scc mod diff	++
16	533/15	65/F	?Ca tongue	0	0	No evidece of malignancy	-
17	561/15	65/F	?Ca buccal mucosa	0	5	Scc - minimally invasive	+
18	568/15	51/M	Growth - hard palate	25	10	Scc - well diff	+++
19	606/15	47/F	?Ca alveolus	0	0	Ulcer with granulation tissue	-
20	655/15	75/M	Growth - alveolus	3	5	Focal carcinoma in situ with adjacent area showing moderate to severe oral epithelial dysplasia	-
21	665/15	45/M	?Ca tongue	3		Scc - mod diff	++
22	672/15	62/M	Growth - hard palate	6	5	Scc - mod diff	++
23	676/15	60/M	Growth - tongue	8	10	Scc - mod diff	++
24	680/15	62/M	Growth - alveolus	20	15	Keratinising invasive scc	+++
25	711/15	29/M	Swelling -cheek	0	5	Florid inflammatory granulation tissue with exudate	-
26	724/15	57/F	?Ca cheek	0	6	Scc - mod diff	++
27	735/15	70/F	Growth - buccal mucosa	0	5	Scc - mod diff	++
28	736/15	57/M	Growth - maxilla & hard palate	6	12	Scc - mod diff	++

S.NO	HPE NO	AGE/SEX	HISTOPATHOLOGICAL SPECIMEN	NO OF CIGARETTES/BIDIS SMOKED/DAY	NO. OF SACHETS OF PAN, GUTKHA OR BETEL QUID CHEWED/DAY	HISTOPATHOLOGICAL DIAGNOSIS	p53 STAINING INTENSITY
29	740/15	65/M	Ca anterior 1/3 tongue	12	8	Scc - mod diff, invasive	+++
30	745/15	65/F	Growth l cheek	0	8	Scc - mod diff	++
31	847/15	70/F	?Ca cheek	0	0	Ulcer with granulation tissue with mild dysplasia, no evidence of malignancy	-
32	849/15	68/M	Growth soft palate	15	6	Poorly differentiated carcinoma soft palate	+++
33	855/15	60/M	Growth - anterior 2/3 tongue	10	8	Scc - well diff invasive	+++
34	882/15	37/M	Growth - cheek	0	3	No evidece of malignancy	-
35	886/15	63/M	?Ca tongue	12	5	Scc - keratinising invasive	+++
36	1021/15	70/F	Ulcer - r tongue	0	0	Ulcer with florid pseudoepithelomatous hyperplasia	-
37	1043/15	47/M	Growth - hard palate	0	0	No evidece of malignancy	-
38	1114/15	60/F	?Ca cheek	0	8	Scc - mod diff	+++
39	1117/15	33/F	Growth - hard palate	0	0	Suggestive of fibrous epulis	-
40	1118/15	57/M	?Ca tongue	8	3	Scc - well diff	+++
41	1138/15	57/M	Buccal mucosa ?ca cheek	5	6	Scc - well diff	+++
42	1292/15	70/M	?Ca tongue	5	3	Hemangioma with thrombi	-
43	1293/15	50/M	Alveolus	8	15	Scc - mod diff	++
44	1294/15	64/M	Ulcer floor of mouth	10	8	Scc - mod diff	++
45	1295/15	37/M	Buccal mucosa	6	6	Scc - mod diff	-
46	1330/15	76/M	Growth- floor of mouth	8	6	Carcinoma in situ	++
47	1424/15	75/F	Ca r cheek	0	6	Verrucous carcinoma	+++
48	1476/15	70/M	Swelling toungue	5	4	Benign squamous papilloma with coexistent hemangioma	-
49	1492/15	66/M	Ulcer tongue	3	3	Scc - keratinising invasive	+++
50	1569/15	80/F	Ca r alveolus	0	10	Scc - mod diff	++
51	1570/15	65/F	Growth - lower lip	0	5	Ulcerated and dysplastic squamous epithelium	-
52	1575/15	55/F	Swelling cheek	0	3	Ulcer with inflammatory granulation tissue, no evidence of malignancy	-
53	1583/15	76/M	Growth cheek, growth hard palate	10	3	Cheek - carcinoma in situ, hard palate - keratinising scc	+++
54	1628/15	55/M	Growth soft palate	8	6	Scc - mod diff	++
55	1636/15	53/M	Ca palate	3	0	Fibroepithelial polyp	-
56	1645/15	52/F	Growth tongue	0	4	Florid inflammatory granulation tissue with pseudoepitheliomatous hyperplasia	-
57	1650/15	70/M	Ca cheek, ca hard palate	12	0	Cheek & hard palate - mod diff scc	++
58	1743/15	66/M	Hemiglossectomy	12	10	Scc - keratinising invasive	+++
59	1745/15	55/F	Ca cheek	0	8	Scc - mod diff	++
60	1813/15	50/M	Whitish discolouration tongue	20	6	Scc - mod diff, invasive	++