DETECTION OF HUMAN PAPILLOMA VIRUS INFECTION IN CASES OF ORAL CARCINOMA USING p16 IMMUNOHISTOCHEMISTRY

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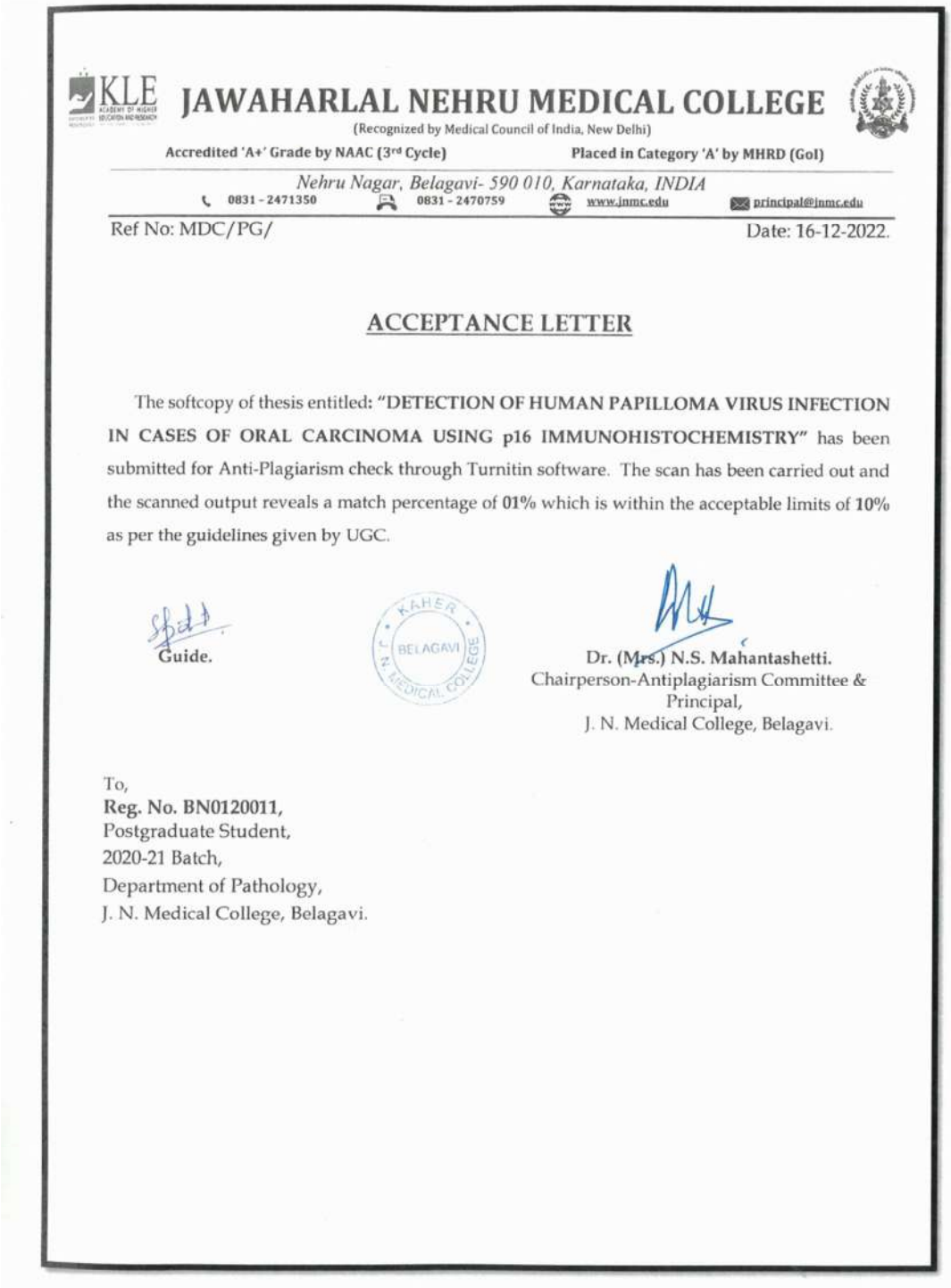

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Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"DETECTION OF HUMAN PAPILLOMA VIRUS INFECTION IN CASES OF ORAL CARCINOMA USING P16 IMMUNOHISTOCHEMISTRY",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Smita Sonoli)
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LIST OF ABBREVIATIONS USED

HPV	-	Human papilloma virus
IHC	-	Immunohistochemistry
HNSCC	-	Head and Neck Squamous cell carcinoma
JE	-	Junctional Epithelial
GTS	-	Glossotonsillar sulcus
SCC	-	Squamous cell carcinoma
PNI	-	Perineural invasion
T	-	Tumour
RMT	-	Retromolar trigone
EMA	-	Epithelial membrane antigen
CDK	-	Cyclin- dependent kinase
PCR	-	Polymerase chain reaction
ISH	-	In situ hybridization
TNM	-	Tumour, nodes and metastases
AJCC	-	American Joint Committee on Cancer
OSCC	-	Oral Squamous cell carcinoma
WHO	-	World Health Organisation
CDKN2A	-	Cyclin- dependent kinase inhibitor 2A
OPSCC	-	Oropharyngeal Squamous cell carcinoma
CK	-	Cytokeratin
EGFR	-	Epidermal Growth factor receptor
RT	-	Radiotherapy
EBRT	-	External beam radiation therapy
CRT	-	Chemoradiation therapy

IMRT	-	Intensity modulated radiation therapy
OSF	-	Oral submucous fibrosis
H&E	-	Haematoxylin and eosin
DPX	-	Dibutylphthalate Polystyrene Xylene
GBS	-	Gingivobuccal sulcus
GCO	-	Global Cancer Observatory

ABSTRACT

"DETECTION OF HUMAN PAPILLOMA VIRUS INFECTION IN CASES OF ORAL CARCINOMA USING p16 IMMUNOHISTOCHEMISTRY"

Introduction- Oral cancer is a major worldwide health issue with high prevalence and fatality rates throughout the world. It is the 6th most prevalent cancer worldwide. Oral squamous cell carcinoma accounts for 40-50% of all malignancies in India. The primary known risk factor for oral cancer includes tobacco and alcohol consumption. Recently Human papilloma virus (HPV) infection of high risk type has become evident and is linked to the development of oral cancers etiologically.

Objectives- To detect human papilloma virus infection in cases of oral carcinoma using p16 immunohistochemistry and to determine the association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry.

Material and methods- A one year prospective study of 50 cases of oral squamous cell carcinoma (OSCC) was taken and histological grading was done as per modified Broder's grading system. All the OSCC cases were studied for p16 expression. Cases showing p16 positivity and its association with histological grading were studied. P value of <0.05 was considered statistically significant.

Results- p16 was positive in 48% of cases with male preponderance. There was no statistically significant association of human papilloma virus infection with the histological grading of OSCC using p16 IHC (p value- 0.304)

Conclusion- p16 IHC is an important biomarker, particularly for high-risk HPV types infection, which makes it useful in evaluating HPV associated squamous carcinoma. Knowing the HPV status by IHC is a more feasible, easy, cost effective, reproducible

test and could impact patient management and survival and the development of prophylactic vaccines based on their viral capsids.

Keywords- Oral carcinoma, Human papilloma virus, p16 Immunohistochemistry

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INTRODUCTION

Oral cancer is a major worldwide health issue with high prevalence and fatality rate throughout the world. It is the 6th most prevalent cancer worldwide[1-4]. The prevalence is higher in underdeveloped nations than in developed nations. In India, oral squamous cell carcinoma is the most frequent form of head and neck cancer, accounting for 40-50% of all malignancies[2]. Squamous cell carcinoma has been seen in a variety of oral anatomical locations. They are listed as follows: lip, tongue, buccal mucosa, lower gingiva, upper gingiva, hard palate, and floor of the mouth[3]. Tobacco and alcohol intake is the most important known risk factors for oral cancer. Recently Human papilloma virus (HPV) infection of high risk type has become evident and is linked to the development of oral cancers etiologically. HPV is a key risk factor in ruling out while assessing a case of oral cancers and is highly associated with oral sex behaviour. These viruses are considered to be a carcinogenic infectious agents not only in cervical cancer but also in a proportion of oral cancers[4]. The most commonly detected high-risk types are HPV16 and HPV18[2]. In oral cancers, HPV appears in as an early initiator of proliferation in the early stage of carcinoma. The viral oncoproteins E6 and E7 associated with HPV cause cancer by binding to and inactivating the tumour suppressor proteins p53 and pRb (retinoblastoma protein). It affects cell control of transcription, thereby promoting the malignant transformation of HPV infected cells leading to p16 overexpression, which is easily detected by immunohistochemistry (IHC)[1].

The human papillomavirus (HPV) was first discovered in 1956, and it was linked to relatively harmless tumours at the time[5]. Later research established the link between HPV and numerous human cancers. The first person to establish a link

between HPV and cancer biology was Harald zur Hausen forty years ago. In 2008, his research into the link between HPV and cervical cancer earned him the Nobel Prize in Physiology or Medicine. Following that, research also found anogenital cancers like penile, anal and vulvar were also linked to HPV which is caused by the sexual transmission route. High-risk HPV has been identified in oropharyngeal and other Head and Neck Squamous Cell Carcinomas (HNSCCs) in recent years[6]. Previously the oral cancer causes includes mainly tobacco users and alcohol users[4].

HPV positive HNSCC has association between sexual behaviour and the disease as compared to HPV negative HNSCC[7]. Up to 80% of cases of HNSCC are ascribed to tobacco use as the primary cause. Both Tobacco and alcohol usage acts synergistically to enhance the risk of HNSCC. While HPV infection has been linked to a significant increase in HNSCC incidence, declining tobacco use has been linked to a decrease in the cancer's prevalence of the same[8]. The tumor suppressing gene remains inactive in HPV infected HNSCC due to genomic alteration caused by the E6 and E7 viral oncoproteins[9].

Hence, this study was aimed at detecting HPV using the p16 immunomarker and its association with oral carcinoma.

OBJECTIVES

1. To detect human papilloma virus infection in cases of oral carcinoma using p16 immunohistochemistry.
2. To determine the association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry.

REVIEW OF LITERATURE

EMBRYOGENESIS-

The head and neck are developed from branchial or pharyngeal arches which appear in week four and five of embryonic development. The mesenchymal tissue forms the branchial arches and they are separated by a deep cleft called as branchial cleft. Pharyngeal pouches are also formed at the same time, although they do not connect directly to the external branchial clefts. As the internal pharyngeal gut develops pharyngeal pouches form along its lateral wall[10].

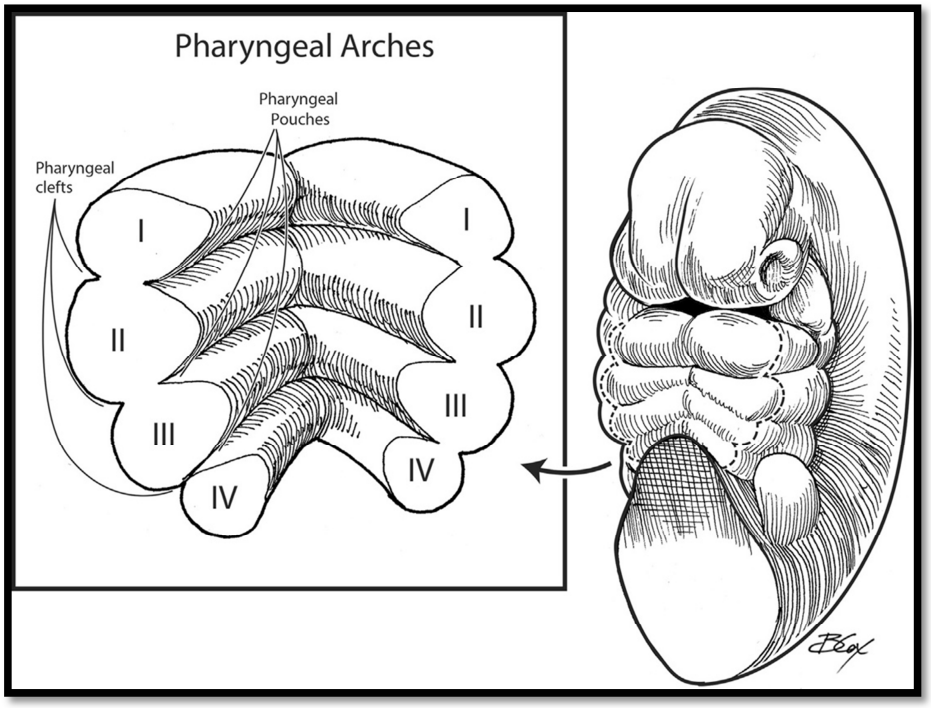


Figure 1- Pouch and cleft formations in the pharynx

(Image taken from Magreni A, Jason G. Embryology of the oral structures. Operative Techniques in Otolaryngology. Head and Neck Surgery. 2015;110-114)

The Oral cavity structures arise from the 1st branchial arch. The 2 maxillary, 2 mandibular and frontonasal processes are visible by the end of 4th week of development. Between 6 and 12 weeks of gestation, the midline fusion between the face and the palate is completed and the upper lip fuses by 6 weeks of gestation. The anterior part of the hard palate is formed during 6 to 10 weeks of gestation. During week 6 of development, the palatine shelves descend on both sides of the tongue after emerging from the maxillary prominences.

During the seventh week of development, the palatine shelves above the tongue become horizontal and unite to produce the secondary palate.

The epithelium of the second pharyngeal pouch gives rise to the palatine tonsil. In the third and fifth months of development, lymphatic tissue invades the primitive palatine tonsil.

At the end of week 4 of development, the tongue is formed by three lingual swellings. The two lateral lingual swellings and one medial lingual swelling, which is called the tuberculum impar. The second branchial arch gives rise to these three swellings.

Due to the outgrowing part of the tuberculum impar, the two-thirds of the anterior part of the tongue is enlarged. A terminal sulcus, in the form of a shallow V-shaped groove, separates it from the posterior third of the tongue. The second, third, and fourth branchial arches give rise to the root or posterior one-third of the tongue.

The second median swelling, also known as the hypobranchial eminence, is comprised of mesoderm originating from the second, third, and fourth arches. The

epiglottis forms as a third median swelling that develops from the fourth branchial arch's posterior region[10].

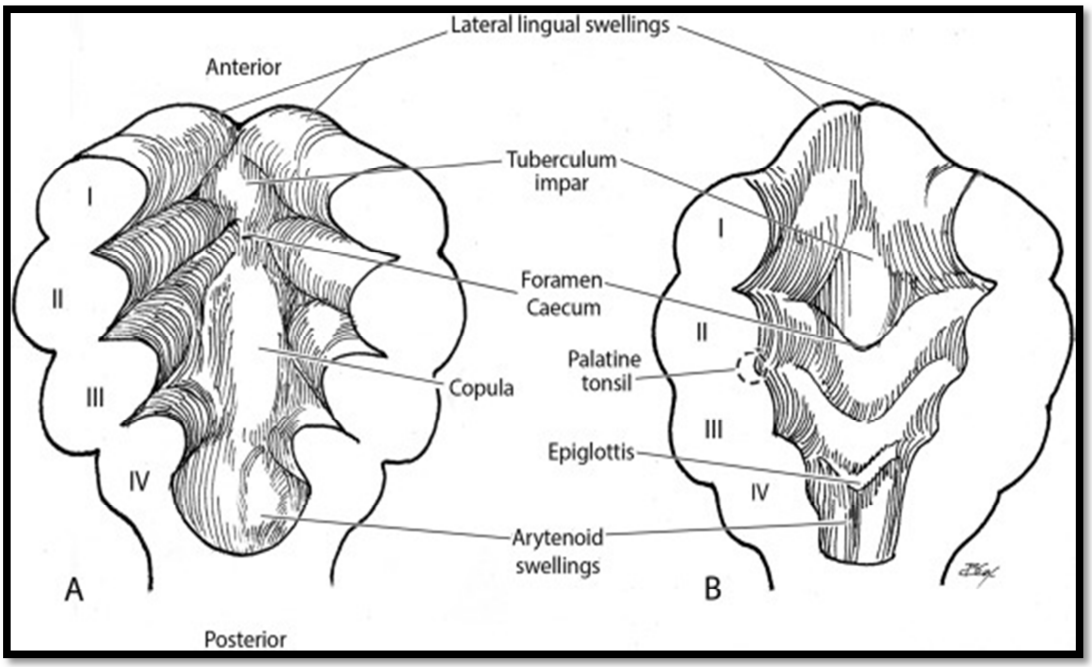


Figure 2- Tongue, palatine tonsils and epiglottis development

(Image taken from Magreni A, Jason G. Embryology of the oral structures. Operative Techniques in Otolaryngology. Head and Neck Surgery. 2015;110-114)

ANATOMY-

The lips are the beginning of the Oral Cavity, which continues as the Oropharynx or the isthmus of the Oropharynx after it reaches the fauces pillars. Oropharyngeal isthmus describes the junction between the oral cavity and the pharynx. It is bounded above by the palatoglossal folds and below by the dorsum part of the tongue[11].

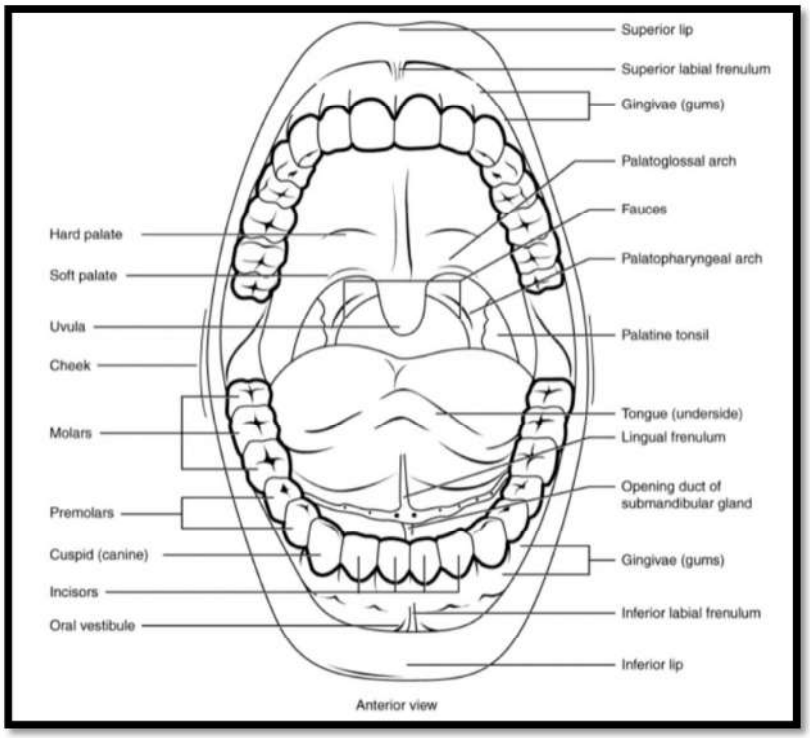


Figure 3- Anterior view of Oral cavity

(Image retrieved on 14.04.2022, from https://www.lecturio./concepts/oral-cavity-lips-and-tongue/)

Oral cavity is divided into Vestibule and Oral cavity Proper.

VESTIBULE-

It is a slit like space between the cheeks and the gums and communicates with the outer surface through oral fissure. There is a vestibule in the oral cavity that is isolated from the oral cavity proper by the alveolar bone and teeth. Reflection of the mucosa covering the alveolus creates a sulcus called the vestibular fornix. Behind the third molar is an opening leading into the main oral cavity from the vestibule. The cheek, which is made up of the buccinator muscle, forms the vestibule's lateral wall. Medially, it is covered with mucous membrane and laterally is the skin[11,12].

ORAL CAVITY PROPER-

The cavity is located within the mandibular and maxillary alveolar borders. The roof of the oral cavity is made up of the anterior hard palate and the posterior soft palate.

The Floor of the Oral Cavity constitutes the Mylohyoid muscle and anterior 2/3rd of the tongue lies on it[13]. Following are the components of the oral cavity:

1. **LIPS-**

The interior surface of the lip is made up of mucous membrane, and its external surface is made up of the orbicularis oris muscle and skin. The vermillion border is the line where the skin and the mucous membrane meet. Superior labial artery and inferior labial artery which are branches of facial artery gives blood supply of the lips. The infraorbital branch of Cranial Nerve V gives sensory nerve supply to the upper lip, while the lower lip is supplied by the mental nerve. The branches of Cranial Nerve VII provide the motor nerve supply[3].

FLOOR OF THE MOUTH-

They are situated between the mandibular medial surface, the tongue's inferior surface, and the mylohyoid. They resemble a tiny horseshoe-shaped area. The mucosa is non-keratinized, which is a hallmark of this condition. The frenulum, a mucosal fold which lies in the midline connects tongue and the floor of the mouth. The sublingual papilla is a large central projection that can be found at the base of the tongue[13].

MYLOHYOID MUSCLE-

It is a triangular and flat muscle near the molars which emerges from the mandible and fuses with the hyoid bone. Its function is to lift the oral cavity and the hyoid bone. It also helps in mandibular depression. Innervation of this region is provided by the mylohyoid nerve, a segment of the trigeminal nerve that originates in the inferior alveolar nerve that runs along the mandible. Blood flow is provided through the mylohyoid branch, or mylohyoid artery, which is a branch of the alveolar artery. The two mylohyoid muscles are combined to make the muscular diaphragm of the floor of the mouth[13].

ISTHMUS OF FAUCES-

This is the region where the oral cavity and the oropharynx join. The palatopharyngeal and palato-glossal muscles serve as its lateral boundaries, superior boundaries by soft palate and inferior by the root of the Tongue[13].

ORAL MUCOSA-

The oral mucosa covers inner surface of the lips, floor of the mouth, base of the tongue, cheeks and alveolar process and gingiva.

It is the mucous membrane that forms the inner lining of the mouth. A combination of stratified squamous epithelium and connective tissue (lamina propria) makes up this structure. It culminates in the retromolar trigone, which is located in the posterior region. The mandibular nerve branch innervates it.

The oral mucosa has the following functions-

The epithelium protects from infection by acting as a barrier between the body and any invading microbes.

Sensation- salivating, swallowing and gagging are the reflexes that are initiated by the oral cavity receptors. Pain, touch, pressure, temperature and taste are the receptors included.

Secretion- the major secretion is saliva produced by the salivary gland but the sebaceous gland secretion is insignificant[12,13].

TONGUE-

It is a striated muscles mass which is covered by the mucous membrane. It has a median septum that splits it into left and right halves.

It has 3 parts-

2. Oral (anterior 2/3rd)
3. Pharyngeal (posterior 1/3rd)
4. Root (base)

Additionally there are two surfaces- dorsal and ventral. A V-shaped sulcus terminalis divides the dorsal surface into anterior two thirds and posterior one third. The foramen cecum serves as a landmark for the sulcus terminalis and the apex of the sulcus faces backward. The anterior 2/3rd mucosa is rough, shows three types of papillae: Filiform, fungiform and Vallate[11,12].

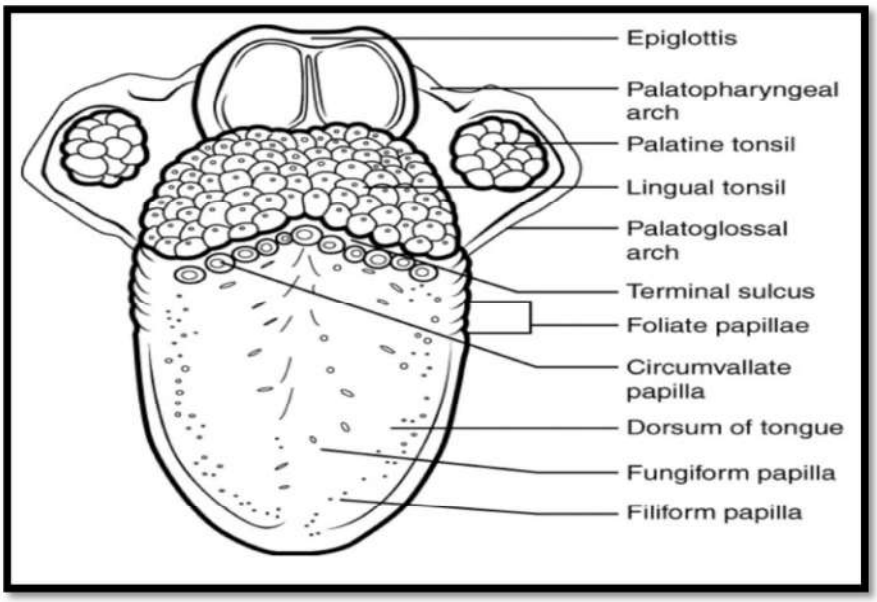


Figure 4- Anatomy of tongue

(Image retrieved on 14.04.2022, from https://www.lecturio./concepts/oral-cavity-lips-and-tongue/)

BLOOD SUPPLY

The lingual artery, a branch from the external carotid artery, provides blood supply to the tongue. The ascending pharyngeal branch of the external carotid and the tonsillar branch of the facial artery gives blood supply to the root of the tongue.

The venous drainage of the tongue involves three venae comitantes. Two of these venae comitantes accompany the lingual artery as it travels through the tongue. The hypoglossal nerve is accompanied by the third venae comitantes as it travels. The Deep lingual vein is the most important and significant vein in the tongue. It is also the largest vein in the tongue. These veins merge to become the lingual vein at the hyoglossus posterior border. These veins drain into the internal jugular vein as their terminal point.

The Tongue's Lymphatic Drainage System:

Submental nodes on both sides receive blood from the tip of the tongue. The anterior two-thirds of the tongue drains unilaterally to the submandibular lymph nodes. Also including the right and the left half remainder of the tongue. Few central lymphatics drain into the same nodes on both sides.

The lymph nodes of the tongue, also known as the jugulo-omohyoid nodes, receive drainage from the posterior one-third of the tongue on both sides. Enlargement of these lymph nodes may be an indicator that tongue cancer is present. The lymph that collects in the upper and lower cervical lymph nodes travels from the most posterior part of the tongue down the sides of the neck[11,12].

RETROMOLAR TRIGONE, HARD PALATE AND GINGIVA

Behind the 3rd molar there lies the Retromolar trigone, which is connected to the maxillary tuberosity at its superior end. The tendinous pterygomandibular raphe, lies behind the retromolar trigone's keratinised mucosa and is attached to posterior mylohyoid ridge and mandibular pterygoid hamulus, into which the buccinator muscle, superior pharyngeal constrictors and orbicularis oris are all inserted. The pterygomandibular space is located between the ascending ramus and the pterygoid muscle, behind the pterygomandibular raphe. This space has both the alveolar and lingual nerves. They have a posterior connection to the deep parotid lobe and parapharyngeal area[3].

The palate is the bony ridge that runs along the roof of the oral cavity and the floor of the nasal cavity. The anterior hard palate and the soft palate are the two parts of the palate. It is the palatine process of the maxilla and the palatine bones that join to form the anterior part of the hard palate. The incisive fossa, along with the two greater palatine fossae and the two lesser palatine fossae, make up the five foramina found in the hard palate. Behind the hard palate is where the soft palate is attached. This posterior layer contains the tensor veli palatini muscle, which is soft and movable. On the underside, there is mucoperiosteum, and at the anterior part of the bone, there are visible transverse ridges. The greater palatine branch of the maxillary artery is the source of the blood supply for the hard palate. The veins that drain from it are part of the pterygoid plexus. The retropharyngeal nodes receive lymph from the hard palate, some of which drains to these nodes but the majority goes to the upper deep cervical nodes[12].

The gingiva is the pink coloured keratinised mucosa also known as gums. It is a part of periodontium, a specialized epithelial tissue which surrounds the teeth via junctional epithelial (JE) cells. The JE serves as a defence against mechanical and microbiological harm and is situated near the base of the gingival sulcus. The mandibular and maxillary branches of the trigeminal nerve provide its nerve supply. The lower gingiva starts at the keratinized mucosa of the mandible and ends at the nonkeratinized mucosa of the floor of the mouth. It is located between the gingivobuccal gutter[14].

PHARYNX-

The oropharynx, nasopharynx, and hypopharynx are all parts of the pharynx.

The epithelium lining the cavities of the oropharynx and hypopharynx are both made up of stratified, non-keratinized squamous epithelium. They have seromucinous glands and lymphoid tissue aggregates which are found in the submucosal compartments.

Nasopharyngeal epithelium is composed of 60% nonkeratinized, stratified squamous epithelium whereas pseudostratified columnar ciliated epithelium lines the remaining 40%. The roof of the posterior wall and the posterior nares are lined with pseudostratified columnar ciliated epithelium. In some areas, the two forms of epithelia appear to have been altered. The mucosa has an intermittent or transitional appearance during the alteration between the two types of epithelia, which may simulate intraepithelial neoplasia.

The blood supply to the pharynx comes from a number of different arteries, including the greater palatine, pharyngeal, and pterygoid branches of the maxillary artery. The dorsal lingual branches of the lingual artery, the ascending pharyngeal branch of the external carotid artery, and ascending tonsillar and palatine branches of facial artery.

On the posterolateral side of the pharynx, Venus connects to form a plexus. Blood flows to the plexus from the pharynx, the soft palate, and the prevertebral region. The veins of the face and internal jugular veins receive its drainage.

The pharynx drains lymph into deep cervical and retropharyngeal nodes. The oropharynx is subdivided into-

1. Glossotonsillar sulcus (GTS) or Tonsillar fossa and Tonsillar fossa

Tonsillar fossa, also known as the tonsillar sinus, is a depression in the lateral wall of the oral cavity formed by the triangular fold (plica triangularis) of the palatoglossal and palatopharyngeal arches. The glottic turbinates and the pharyngoepiglottic fold form the inferior border of tonsillar fossa, while the anterior and the posterior tonsillar pillar define its anterior and posterior boundaries, respectively. Laterally, this area is bounded by the mandible, the lateral pharyngeal space, and the pharyngeal constrictor muscle. Glossotonsillar sulcus, which runs from anterior tonsillar pillar to pharyngoepigglotic fold, separates it from the base of the tongue.

2. Base/Root of the tongue-

It encompasses the posterior third of the tongue and is limited by the tonsillar pillar on the anterior side while the posterior tonsillar pillar acts as a boundary posteriorly. The inferior side is bounded by the pharyngoepiglottic fold and the Glossotonsillar sulcus.

3. Soft palate-

It is the anatomical partition between the nasopharynx and the oropharynx. The soft palate is a slender and very flexible muscle complex.

4. The Posterior pharyngeal wall-

The prevertebral muscles and the pharyngeal mucosa make up the posterior pharyngeal wall. The wall extends from the base of the skull to the cricopharyngeus muscle

5. Palatine Tonsil

Facial artery tonsillar branch is the primary source of the tonsil's arterial supply. Ascending palatine branch of facial artery, Greater palatine branch of maxillary artery, pharyngeal branch of external carotid artery and lingual artery dorsal branches are additional sources of vascular supply.

Drainage of the veins occurs in the palatine, pharyngeal, or facial veins. Lymph flows into the jugulodigastric node, which serves as a lymphatic drainage point[12,15].

HISTOLOGY OF ORAL CAVITY-

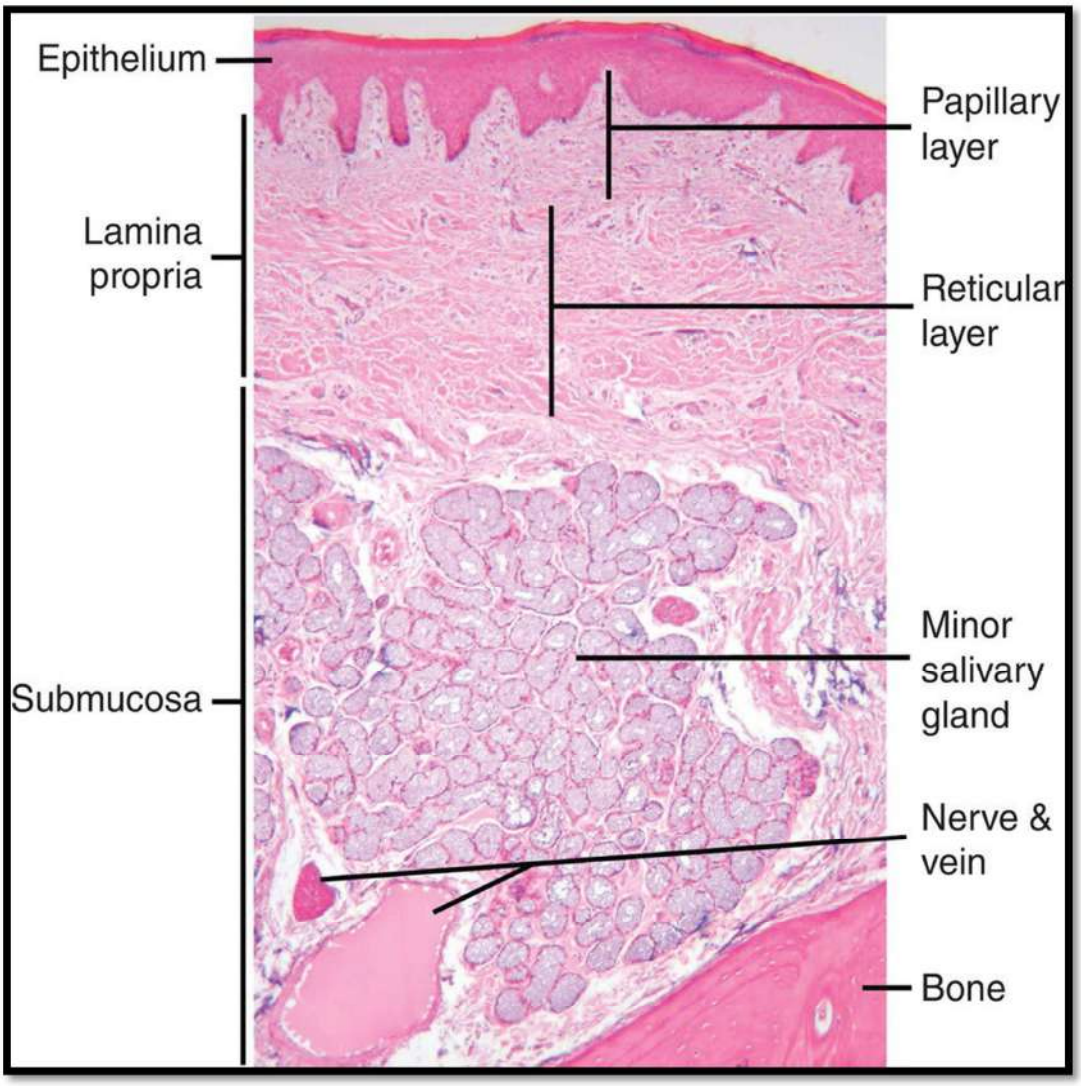


Figure 5- Histology of Oral Mucosa showing all the layers.

(Image retrieved on 15.04.2022, from https://pocketdentistry.com/12-oral-mucosa)

The oral cavity mucosa is lined by stratified squamous epithelium. They are highly organized, avascular and semipermeable tissue. The position in the oral cavity, and the specific mechanical needs of the region influences the thickness and degree of keratinization. Epithelium and lamina propria are connected by an interdigitated interface. Rete pegs are wavy projections that connect to the lamina propria's

underlying papillary projections at the deeper layer of the epithelium. The epithelium is firmly linked to the interstitial basement membrane between these two tissues. On light microscopy, the basement membrane connects them to the connective tissue and provides support to the epithelium and regarded as a line dividing the connective tissue of the lamina propria and the epithelium of the layer underneath it.

Based on histology, clinical characteristics and functional characteristics, the oral mucosa can be divided into three groups. Moveable or lining mucosa can be found in the mobile structures of oral cavity which includes cheeks, alveolar mucosa, vestibular fornix, the lips, soft palate, and floor of the mouth. The mucosa is covered by stratified squamous epithelium that is non-keratinized. The gingiva and hard palate have masticatory mucosa, which is a rigid mucosa that is firmly attached to the underlying bone. Para keratinized or keratinized stratified squamous epithelium is the type of epithelium that covers these areas. With this, the masticatory mucosa is better able to withstand the strain or stress that is placed during mastication.

The dorsum of the tongue has a specialised mucosa that reveals either a nonkeratinized or keratinised stratified squamous epithelium. It is known as specialized because it has a specialised collection of lingual papillae and taste buds that allow for the perception of a variety of tastes. This is also categorised as the masticatory mucosa because of its actively participation in mastication.

The keratinized oral epithelium consists of four layers: the basal layer, also known as stratum basale, the spinous layer, also known as stratum spinosum, the granular layer, also known as stratum granulosum, and the cornified layer (stratum corneum).

The mucosal lining which has non keratinized epithelium has two layers known as the stratum filamentosum and stratum distendum above the stratum basale. There is absence of granular layer in mucosal lining but have thinner layer of stratum spinosum. The squamous epithelium is characterised by intercellular desmosomes connection and a uniform flattening of epithelial cells from the stratum basalis to stratum corneum.

The keratinocytes that make up the majority of squamous epithelial cells are made of cytokeratins.

The stratum basale is comprised of a layer of columnar or cuboidal cells which lies above the basement membrane and they are attached with hemidesmosomes. These cells are renowned for their propensity for mitosis.

The stratum spinosum comprised of multiple layers of bigger cells known as prickle cells and is located above the stratum basale. The stratum granulosum- Small cytoplasmatic keratohyalin granules seen in these cells exhibit a strong hematoxylin stain.

The stratum corneum, also known as the stratum superficiale, is a layer which is keratinized. The layer is formed by flattened cells with no nuclei and appear pink when stained with eosin[16].

Lamina Propria

Underneath the epithelium is a connective tissue layer called the lamina propria, which contains neurons, inflammatory cells, macrophages, mast cells, fibroblasts and blood vessels. All of them are enveloped in an amorphous substance that is composed of glycoproteins and proteoglycans.

The papillary layer at the surface and the reticular layer at the deeper level are the two layers that make up the lamina propria.

Thin collagen fibres that form undulating papillae ridges and join with the epithelium to form the papillary layer. For the transportation of nutrients, this surface offers a larger area. This layer is filled with capillary loops.

Between the papillary layer and the underlying structure (submucosa or the periosteum respectively) lies the reticular layer. Even though the basal fibres eventually align to run perpendicular to the periosteum, this layer is composed of denser collagen fibres that run in a direction parallel to the surface. Because of their firm connection to the bone, and this fibrous attachment the oral mucosa is protected from the shear and compression force and is known as mucoperiosteum.

The fibroblast, which is the main cell in the lamina propria, plays a role in the production of new connective tissue and amorphous substance. They help to repair and maintain damaged tissues. In cases of drug induced gingival overgrowth, the fibroblasts in the gingival tissues are activated and proliferated, leading to an increase in the secretion of amorphous substance like glycosaminoglycan.

During the healing of wounds, macrophages also promote the growth of fibroblasts and primarily engage in phagocytic activities. The connective tissue of the lamina propria contains mast cells.

The cytoplasmatic granules that make them unique contain the anticoagulant heparin and inflammatory mediator histamine, which is responsible for initiating vascular alterations during the process of inflammation. Collagen (type I, III) and elastin are the two main primary fibres in the lamina propria's connective tissue[16].

Submucosa

Underneath the lamina propria is the submucosa. This tissue contains blood vessels and nerves and is made up of elastic and fibrocollagenous tissue. Adipose tissue, lymphoid tissue, minor salivary glands, and muscle depending on the location, may also be present.

The submucosa lines all parts of the buccal cavity beside the masticatory mucosa-covered hard palate and accompanying gingiva, where there is no submucosal layer and the lamina forms a mucoperiosteum over the underlying bone. The submucosa of the oral mucosa is occasionally the site of ectopic sebaceous glands or Fordyce granules.

Fordyce granules have traditionally been thought to be a normal variation, but recent research suggests that individuals with higher lipid profiles have more of them. In light of this, we cannot dismiss the clinical finding. The buccal mucosa and labial mucosa are where they are most commonly found[16].

Histology of Lip-

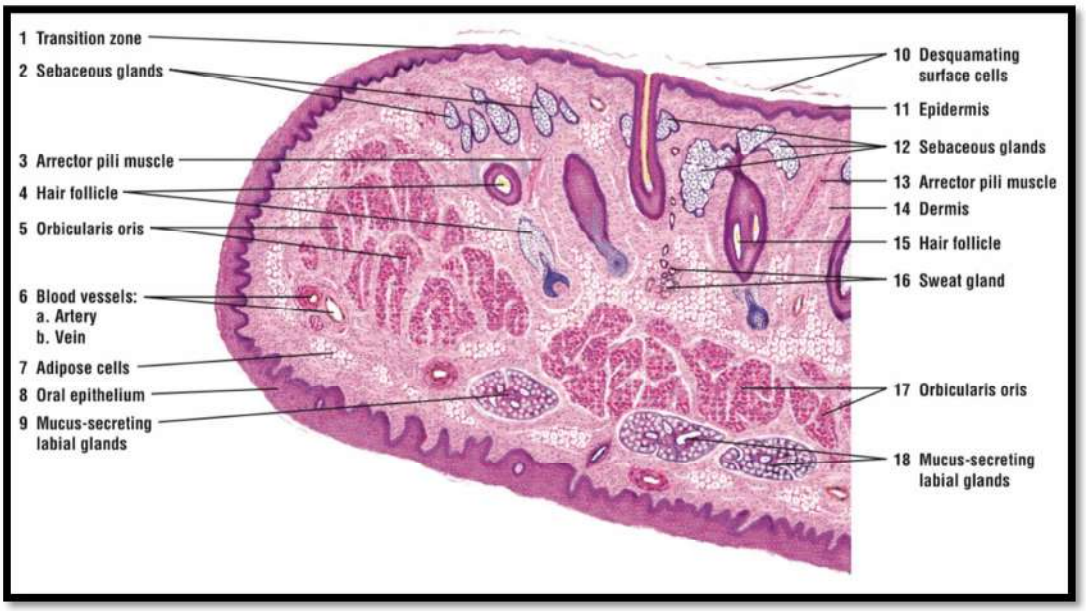


Figure 6- Lip (longitudinal section)- H&E, Low magnification.

(Image taken from DiFiore`s Atlas of Histology with Functional Correlations. 11th ed. South Asian, Lippincott Williams & Wilkins; 2010)

The outer lip is lined by a thin layer of epidermis made up of desquamating surface cells on a stratified squamous keratinized epithelium. The dermis, sebaceous glands that are associated with hair follicle lies beneath the epidermis. A deeper layer of the dermis contains simple tubular sweat glands. The dermis contains smooth muscles that attach to hair follicles known as arrector pili muscles. The orbicularis oris is a layer of striated muscles that makes up the core of the lip. The mucocutaneous junction is the interface between the epidermis of the skin and the oral epithelium. The oral epithelium which lines the internal surface of the lip is a moist, stratified squamous nonkeratinized epithelium. It is a layer that is thicker than the epithelium of epidermis. The tubuloacinar labial glands, which secrete mucus, are located in the deeper connective tissue and their secretions helps in moistening the

oral mucosa. There are numerous capillaries, blood vessels, and fat cells present in the lip's underlying connective tissue[17].

Histology of Tongue-

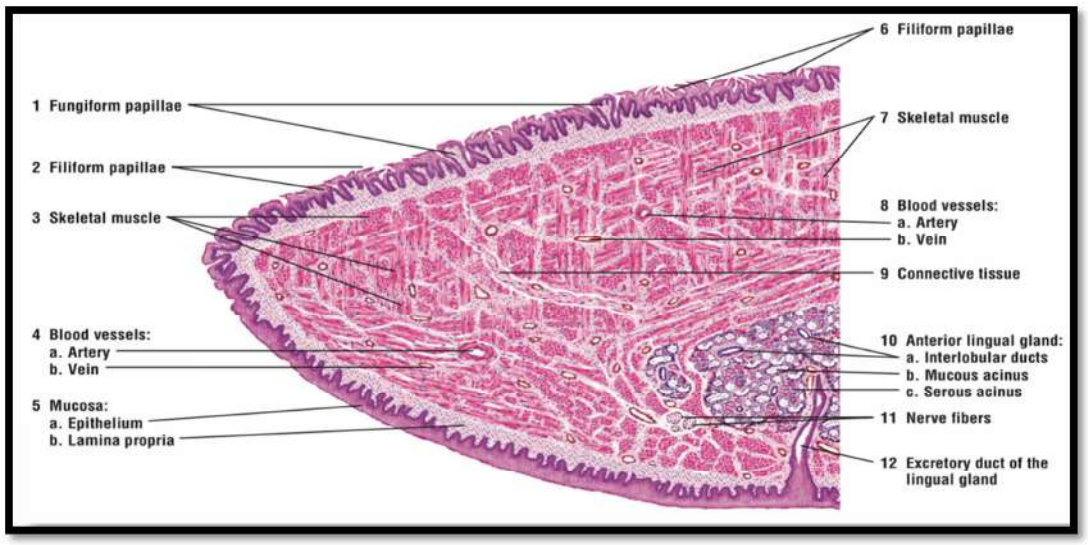


Figure 7- Anterior region of the Tongue (longitudinal section)- H&E, Low magnification.

(Image taken from DiFiore`s Atlas of Histology with Correlations. 11th ed. South Asian, Lippincott Williams & Wilkins; 2010)

The tongue's dorsal surface is rough and characterised by numerous mucosal projections called papillae which are in contrast to the mucosa of ventral surface which is smooth. All of the dorsal surfaces are covered with slender, conical shaped filiform papillae, and tips of filiform papillae show partial keratinization and few are fungiform papillae. It has a substantial lamina propria core and a broad, rounded surface of non-cornified epithelium. The core or central structure of the tongue are formed by intertwined skeletal muscle bundles. Blood vessels and nerve fibres are found in the connective tissue that surrounds these muscle bundles. A portion of

anterior lingual gland lies in the lower half of the tongue. This gland is a mixed form, containing both mucinous and serous acini. The interlobular ducts enter into the bigger excretory duct of the lingual gland and is located on the ventral surface of the tongue which opens into the oral cavity[17].

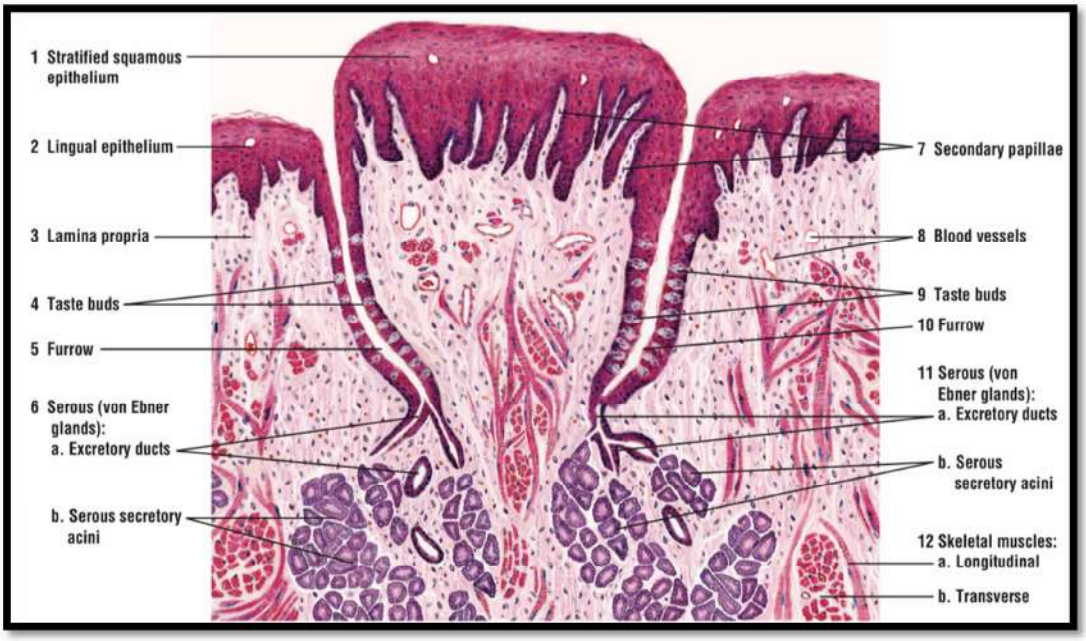


Figure 8- Cross section of a posterior tongue: serous (von Ebner`s) glands, surrounding furrow and H&E, Low magnification.

(Image taken from DiFiore`s Atlas of Histology with Functional Correlations. 11th ed. South Asian, Lippincott Williams & Wilkins; 2010)

The stratified squamous epithelium of the tongue covers the circumvallate papillae and is known as the lingual epithelium. Numerous secondary papillae emerge from the lamina propria and project into the stratified squamous epithelium that covers the papilla. Each circumvallate papilla has a deep trench or furrow at its base. The circumvallate papilla's lateral surface and the outer wall of the furrow contain the oval taste buds. Numerous tubuloacinar serous glands are located in the midline of the tongue as well as in lamina propria.

The tongue's motion for phonation, chewing, and swallowing is made possible by the skeletal muscles that weave throughout its centre. Many blood vessels are found in the lamina propria[17].

PATHOLOGY AND PATTERNS OF SPREAD-

LIPS-

The most prevalent type of tumour in the lip is squamous cell carcinoma (SCC). Leukoplakia and carcinoma in situ are commonly seen in lower lip and it may occur several years before the carcinoma does. In 2% of cases, there is a perineural invasion (PNI) which was seen in conjunction with a large size tumor, recurrent lesion, with histological differentiation of poorly type and mandibular invasion. Internal jugular chain lymph nodes, as well as submental and submandibular lymph nodes, are affected by lymphatic metastasis[3].

FLOOR OF THE MOUTH-

The most prevalent neoplasm is squamous cell carcinoma (SCC), a moderate level of impairment. 5% of malignant tumours in this region may be of Adenoid cystic and mucoepidermoid carcinoma. Primary tumour metastasizes to this area. Roughly 90% of malignant tumours originate from the anterior midline part of the floor of the mouth within 2 centimetres. They enter the genohyoid and genioglossus muscles after penetrating the submucosa to reach the sub lingual gland. Until the lesion becomes advanced, the myelohyoid muscle acts as a barrier. The tumour expands early into the gingiva and periosteum of the mandible and spreads along periosteum rather than through it. The posterior extension affects the muscle at the tongue's base. Mandible invasion is seen as late manifestations. The submandibular

gland becomes painful, and firm and frequently enlarges when the duct is obstructed. In about 30% cases, clinically positive lymph nodes are seen and in 4% instances bilateral nodes are affected. Risk for occult metastasis is 10-15% in lesions with T stage of 1 or 2. Here, level IB and II nodes are frequently implicated in high risk bilateral spread[3].

TONGUE-

Most oral tongue tumours affect the lateral and ventral tongue surfaces. Early diagnosis is possible for the anterior two-thirds of the tongue. In advanced cases, the base/root of the tongue and the floor of the mouth are invaded, resulting an ulceration and fixation. A lesion in the posterior third of the tongue is seen towards the root of the tongue and the tonsillar pillar anterior part. Invasions of the blood vessel and peri neural invasion are not uncommon. Levels IB and II are the earliest to show evidence of lymphatic spread and seldom involvement of submental and level V is also seen. There will be clinically positive nodes in about 35% of the patients and bilateral nodes positive is seen in 5%. Incidence for occult metastasis is approximately 30%. As the T-stage progresses, so does the percentage of lymph nodes that are positive also increases[3].

BUCCAL MUCOSA-

Most malignant tumours in this area are low-grade squamous cell carcinomas, which often appear against a leukoplakia or lichen planus background. Likewise, verrucous carcinoma may also develop. Melanomas and tumours of the salivary glands are rarely seen here. The lesions appear to be discrete and exophytic in the early stage. The buccinator muscle provides a clear resection margin if there is tumour negative and also functions as natural barrier to stop the further spread. The

underlying muscles, skin, gingivobuccal sulci, gingiva, and bone are all invaded by the tumour as it spreads. Level I and II lymph nodes are involved in the initial stages of lymphatic dissemination including the parotid nodes. The incidence of about 9-31% of lymph node positivity is observed. About 16% of people are at risk for occult disease[3].

RETROMOLAR TRIGONE, HARD PALATE AND GINGIVA

The most prevalent type of tumour is squamous cell carcinoma (SCC). Among the tumours of salivary gland, adenoid cystic type of carcinoma commonly affects the posterolateral side of hard palate. It is the lower gingiva that typically displays the verrucous cancer along with few reports on melanoma cases. The odontogenic epithelium or the embryonically trapped epithelium may give rise to SCC within the mandibular body or maxilla. Also, more commonly seen in molar regions. Identifying ameloblastoma from metastatic SCC is crucial. An Ameloblastoma is an odontogenic tumour which is benign and affects both maxilla and mandible. They can be aggressive locally but is otherwise quite uncommon with an incidence of about 1%. It is more commonly seen in mandible.

In the lower gum, the SCC spreads to periosteum, floor of the mouth and the buccal mucosa nest to them. It is usual for low grade lesions to first manifest as a smooth, saucer-shaped lesion before spreading to the mandible. Lesions of moderate to high grade tend to invade bone. Levels I and II lymph nodes have been infected by the tumour. Between 18% and 52% of nodes are positive. Occult disease in approximately 17-19%. A rare malignant type of Ameloblastoma called Ameloblastic carcinoma, has potential to spread the lymph nodes in the area and to other parts of the body.

Squamous cell cancer (SCC), which develops in the hard palate and upper alveolar ridge, comes from gingiva. The soft palate, hard palate, buccal mucosa and underlying bone all become secondary sites for dissemination. The sinus invasion in maxillary part can be seen if there have recently been extractions that have provided access. 13-24% positive lymph nodes risk is seen at diagnosis. It is estimated that 22% of all illnesses are not easily diagnosed.

In the retromolar trigone (RMT), as the SCC progresses it invades the neighbouring tissues and eventually reaches the buccal mucosa, anterior tonsillar pillar and buccal mucosa. Pterygomandibular and medial pterygoid muscle are both infiltrated by the posterior metastasis. Due to the extremely thin mucosa covering the bone, mandible invasion occurs early. Buccinator muscle and fat pad are invaded by posterolateral spread of the tumours.

Dissemination via the lymphatic system is initially detected in the level I and level II lymph nodes. A clinical examination will reveal positive nodes in approximately 30% of patients. Occult disease risk ranges between 15 and 25 percent[3].

ORAL CAVITY SQUAMOUS CELL CARCINOMA-

The base of the tongue and the tonsils are frequent locations for oral cavity cancers. Typically these tumours appear solid and lack differentiation resembling large cell malignant lymphoma. Dysplastic alterations can be seen in the epithelium that surrounds the tumour[18]. Eosinophilic infiltration is related with a favourable prognosis in the SCC of oral cavity[19]. If immunohistochemical markers are checked, it is likely that the perineural and vascular invasion of SCC has occurred[20].

Different variants of Squamous cell carcinoma (SCC)

1. Verrucous Carcinoma

Grossly, it appears as a large, soft papillary growth that is fungating in the oral cavity. They are frequently infected and spread into the cheek's musculature. They penetrate the maxillary or mandibular region and perineurial tissues are invaded. Invasion into the nodes is rare in this carcinoma.

Microscopically, identifying a verrucous carcinoma might be challenging because they are well differentiated. A superficial biopsy will only diagnose a benign papillomatosis. Having a good proper biopsy will show rete pegs with a complicated pattern, which are swollen and voluminous and extend into the deeper tissues. The tumour's well-differentiated cells set it apart from squamous cell carcinoma. Compared to squamous cell carcinoma, verrucous carcinoma cells are larger. Squamous cell carcinoma can be found in the foci of 20% of patients with verrucous carcinoma. There is a high potential for recurrence of this kind[21].

Polypoidal growths can be seen on the surface of the larynx, and close examination with a microscope reveals that these growths are highly differentiated. They tend to spread locally rather than distant metastasis. The two conditions, verrucous hyperplasia and carcinoma seem very similar. It is challenging to identify both conditions with a small biopsy and they are classified based on the presence or lack of invasion. Laryngeal cancer is a type of carcinoma that can also be a hybrid. The treatment entails surgical intervention. When an anaplastic transition occurred, radiation therapy is the treatment[22].

2. Adenoid squamous cell carcinoma/ Pseudoglandular

Actinic radiation is the root cause of this type of cancer and the primary location is the lip. Actinic radiation has no role in the development of these lesions in the gingiva or the tongue, however, they may occasionally manifest. Their appearance is either alveolar or pseudoglandular pattern[18,23].

3. Adenosquamous carcinoma

These tumours are quite uncommon. Amidst the actual glandular differentiation, squamous differentiation also occurs. While certain tumours can originate in the small salivary glands but they are distinct from carcinomas of the mucoepidermoid[24,25].

4. Basaloid Squamous cell carcinoma

It poses the greatest risk in the oral cavity. The larynx, oral cavity, and esophagus are the common sites of infection. Rarely, they can manifest in the lungs. Anal canal cloacogenic carcinoma may have similar characteristics with basaloid cancer. Similarities with adenocarcinoma are subtle. Their microscopic appearance is distinct from that of adenosquamous carcinoma, although on a histogenetical basis, they resemble adenosquamous carcinoma. There is squamous differentiation and mixed solid tumour in basaloid cancer. Basaloid carcinomas typically exhibit a palisading pattern at their periphery and a substantial basement membrane.

They resemble adenoid cystic carcinoma in their cystic areas. These cystic cavities are filled with mucoid and hyaline material. The prominent appearance of basal lamina is the hallmark of this tumour. This is shown both ultra-structurally and

immunohistochemically. High molecular weight keratin, as identified by the 34Eβ12 antibody, is expressed by tumour cells.

In the larynx, it takes a similarly belligerent path. Heavy smokers are more likely to get affected with this lesion. The hallmark features include hyperchromatic nuclei, less cytoplasm, necrosis and peripheral palisading pattern of the tumour along with an attempt made to distinguish towards glandular structures. The pharynx, esophagus and tongue are also possible locations for this type of tumour. Contrast with adenoid cystic carcinoma, which is a different disease[26].

5. Spindle cell (sarcomatoid) carcinoma

They typically develop as a polyp or an infiltrative growth that affects the lip, tongue or other oral cavity's area and they are generally ulcerated. The sarcoma-like lesions are frequently found in conjunction with squamous cell cancer. The recurrence of spindle cell carcinoma is possible after the initial diagnosis of SCC[27]. The recurrence along with the evidence of electron microscopy, immunohistochemistry and molecular biology all points to the sarcomatoid component being the result of a metaplastic transformation in original epithelial tumor. Immunostaining is useful in distinguishing between sarcomatiod from sarcoma. Epithelial membrane antigen (EMA), p63 and E- cadherin (epithelial cadherin in the membrane) are the immunostains used. Malignant fibrous histiocytoma is very similar to the sarcoma like part. Mesenchymal differentiation along the muscle fibre trajectories might be visible in this type of carcinoma. Larger cells cytoplasm is filled with hyaline globules. The prognosis is conditional on the degree of invasion.

Polypoidal growth is the most common form of the laryngeal sarcomatoid tumour and is similar with a laryngeal polyp. Under the microscope, they look like a combination of squamous cells and sarcoma. In certain cases, the sarcomatoid component can be mistaken for granulation tissue, whereas in others it can resemble osteosarcoma or giant cell tumour of malignant origin and malignant fibrous histiocytoma[28].

6. Papillary squamous cell carcinoma

It typically affects the oropharynx of elderly people and is HPV-positive. This growth occurs as an exophytic outpouching of the larynx. Human papillomavirus (HPV) is the most common cause, and the prognosis is favourable in comparison to that of sinonasal cancer. Cytological atypia distinguishes it from verrucous cancer[29].

The Risk factors and Pathogenesis for HNSCC-

Similar to HPV- positive cervical SCC, HPV- positive HNSCC shares the same risk factors. HNSCC patients who test positive for HPV tend to be younger age group and have lower rates of cigarette and alcohol use[30].

Both human papilloma virus- positive and negative HNSCC share a molecular profile that is comparable to that of cervical squamous cell carcinoma which is HPV positive. HPV-positive cervical cancer patients tend to have comparable demographics, including younger age, earlier age of first intercourse, higher rates of oral sex, and higher rates of sexual partners[31].

Table 1: The Risk factors[32]

The HPV- positive and negative risk factors for Oropharyngeal cancer	
HPV positive Oropharyngeal cancer	**HPV negative Oropharyngeal cancer**
1. Number of oral sex partners 2. Many vaginal sex partners 3. Young age at first sexual contact 4. Anogenital warts	1. Consumption of Nicotine 2. Consumption of alcohol 3. Older age 4. Poor oral hygiene

Factors increasing the chance of contracting Human papilloma virus includes cigarette smoking, excessive alcohol use, and sexual behaviour. These are the 3 main causes of HNSCC, however many other variables contribute as well. Eight major carcinomas are directly linked to the tobacco use in a maximum of 13 sites, including HNSCC, according to numerous international health organisations. Tobacco users have a higher fatality rate when compared to non-smokers. As tobacco consumption rises so does the likelihood that it will cause a cancer[33,34].

Alcohol usage is prevalent among the HNSCC patients, according to a study. No research is there to substantiate the independent risk factors for HNSCC of alcohol and cigarette use. When taking into account the risk factors for HNSCC is alcohol, the degree of consumption of alcohol is taken into consideration. No similar study has been reported with daily alcohol consumption, although it is plausible to predict that increasing alcohol drinks may increase the risk of developing HNSCC[35].

HNSCC risk has been linked to tobacco use and alcohol use together. High risk of HNSCC is further increased by concurrent excessive alcohol and tobacco usage[36].

The molecular processes controlling cell cycles are influenced by viruses such as the herpes group, adenoviruses, and HPV. Activation of cellular oncogenes also known as proto-oncogenes can occur through amplification of the gene, point mutations, or rearrangements of other gene[37].

The herpes virus family and HPV are now recognised as synergistic viruses that contribute to many HNSCC. From both benign and malignant neoplasms, over a hundred different genotypes have been isolated. HPV 16 and HPV 18 have the highest rates of genotype isolation. They are also present in the oral mucosa of healthy individuals. The tumour-suppressing gene is rendered inactive by HPV oncoproteins E6 and E7, which cause genomic alterations in HNSCC[38,39].

Immunocompetence is primarily influenced by the E6 and E7 HPV oncoproteins, which modifies the intercellular immunological system. Human papilloma virus (HPV) oncoproteins have the ability to bind with a wide variety of human gene products, including those involved in cell proliferation and differentiation regulation mechanism[40].

Molecular pathways of Human papilloma virus induced Cancer

The human papillomavirus is a type of virus that replicates by copying its own DNA double-helix molecule. While over 200 distinct HPV strains have been identified, only a small fraction of them causes human cancer. Many Human papilloma virus (HPV) infect the epithelial cells of the skin or the mouth (non-

mucosal epithelium), however they have varied anatomic site preferences. The HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 were categorised as possibly carcinogenic to humans by the International Agency for Research on Cancer. Cervical cancer and other forms of HPV infection are largely attributable to these HPV strains. In addition, the HPV is subdivided into low and high-risk categories based on the virus's capacity to successfully transition into the host cell. Cervical cancer is caused by HPV 16, 18, 31, and 45 subtypes, which are high risk subtypes. HPV 6 and 11 subtypes are low risk subtypes[41,42,43].

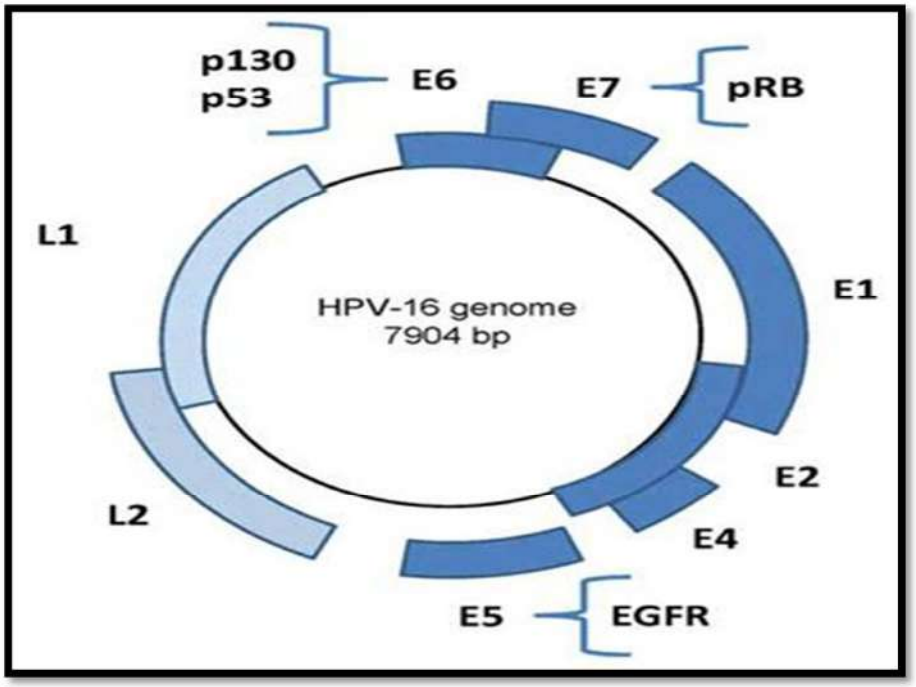


Figure 9- HPV- 16 Genome[44]

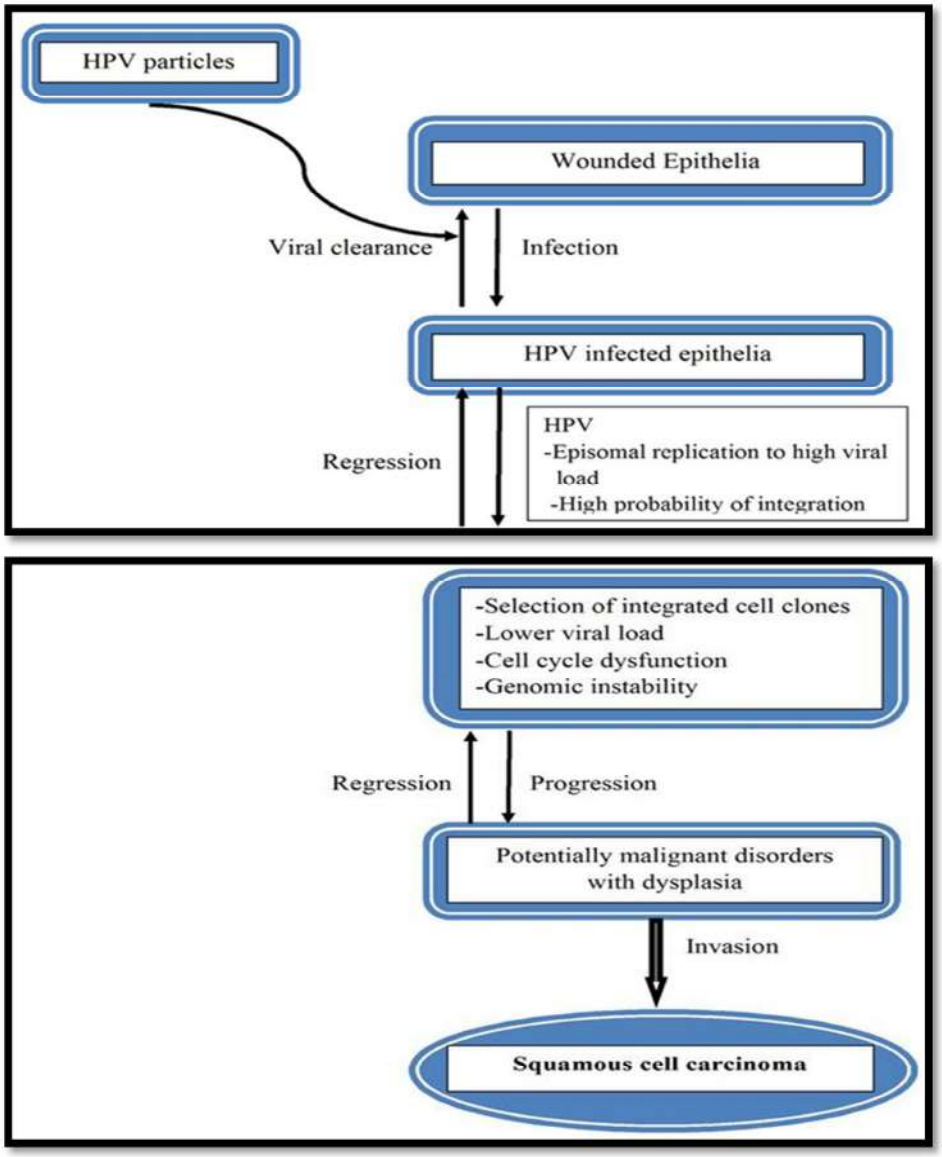


Figure 10- Schematic depiction of the steps that lead to cancer following HPV infection[45].

Table 2: High risk HPV types[46,47]

Highest risk	**16, 18, 31 and 45**
Other high risk	**33, 35, 39, 51, 52, 56, 58 and 59**
Probably high risk	**26, 53, 66, 68, 73 and 82**

There are roughly 8000 base pairs in the HPV double stranded genome. Their structure is composed of three distinct parts: A noncoding long control region, also known as an LCR that regulates the expression and replication of genes. Both the early region, which encodes the proteins E1-E7, and the late region, which encodes the proteins E8-E21, are responsible for encoding proteins that are necessary for gene expression, replication, and survival. Capsid proteins are essential for both the packaging of the viral genome and the subsequent release of the virus, and they are encoded in the late (major L1 and minor L2) area[41]. There are three viral oncoproteins that are encoded by the early genes and these proteins are designated as E5, E6 and E7.E6 and E7 play crucial roles in both healthy and malignant cell division. The HPV 16 E6 and E7 proteins and other high risk types trigger ubiquitin-dependent degradation of tumour suppressor proteins p53 and pRb[48]. It is the E6/E6AP complex, which includes the cellular protein E6AP that regulates p53 protein degradation through ubiquitination and proteasomal destruction. A malfunction of p53 would occur in many human cancers despite the fact that it performs a role in cell protection. p53 mutations is reported in HPV infected cancer where p53 degradation is triggered by E6 oncoproteins[48,49]. Chromosomal instability in cells containing HPV 16 E6 oncoproteins is the first stage in carcinogenesis. The G1 S phase transition, apoptosis, mitosis and differentiation are all regulated by pRb, in which HPV E7 binds to E2F,

rendering it inactive by proteasomal degradation. After E2F is released, Cyclin A and Cyclin E are activated, which causes cells to enter the G1 S phase[50]. Increased induction of $p16^{INK4a}$ to a greater extent is due to deactivation of pRb gene. HPV positive infection lesions and cancer, tumour suppressor $p16^{INK4a}$ is used as biomarker. Over expression of p16 is observed in the majority of the HPV-positive HNSCC. Loss of $p16^{INK4a}$ and p53 due to mutation is common in HPV- negative HNSCC and is largely attributable to tobacco and alcohol use. When Rb is deactivated in cancer cells, oncogenic effect of p16 is mitigated by blocking CDK4/CDK6. Phosphorylation of CDK4/CDK6 substrate in cells where Rb has been inactivated, may result to cell death[51,52,53].

Different Diagnostic methods for HPV positive Oral squamous cell carcinoma-

As of yet, there has been no agreed upon method for identifying HNSCC that has been caused by HPV. Tissues and exfoliated cell samples can be used for testing HPV DNA. Molecular detection is the Gold Standard. The Southern blot test is the one of the old method that is used to detect HPV DNA and has high sensitivity and low false positive rates. It can distinguish different types of HPV specifically. Southern blotting is time consuming and requires huge amounts of cellular DNA, making it unsuitable for routine clinical application. When compared to the southern blot test, the sensitivity of IHC for detecting p16 expression is higher but the specificity is lower[54,55,56,57]. Both the expression of p16 and the genetic components associated with HPV are detected in the tiny tumour samples using polymerase chain reaction (PCR). This technique is extremely sensitive to the presence of viral genomes in oral tissues that have not been linked to cancer. Thus, it creates a significant incidence of false positives. However, this method is useful for detecting oral cancer.

The HPV subtypes are detected using in situ hybridization (ISH), which has a lesser specificity when compared to the Southern blot test[58,59]. Serum HPV- specific antibodies have been linked to an increase risk of developing HNSCC[60].

Table 3: Detection of HPV infection[58]

	METHODS	ADVANTAGES	DISADVANTAGES
DNA	**PCR**	**High sensitivity, Type specific and type overlapping, Formalin fixed tissues acceptable**	**Risk of contamination False positive result possible**
	In situ hybridisation	**Morphologic analysis in tissue slides**	**Low sensitivity Type specific probes**
	Southern blot	**High specificity, Integration visible, Type specific**	**Low sensitivity High DNA consumption**
RNA	**Real-time PCR**	**High sensitivity and specificity Detection of biologic activity**	**Complex procedure Fresh tissue necessary**
	In situ hybridisation	**Morphologic analysis in tissue slides**	**Low sensitivity**
Protein	**E6/E7**	**Easy procedure Detection of biologic activity in fixed tissue samples**	**Fluctuant sensitivity and specificity**
	p16		

CLINICAL FEATURES FOR ORAL SQUAMOUS CELL CARCINOMA (OSCC)-

LIPS- Most prevalent site is the vermilion of the lower lip. They present as enlarge discrete lesion and nontender until it ulcerates.

Leukoplakia or carcinoma in situ are the lesions that are superficial and ulcerated, with little or no mass. The skin around it has erythema, and the skin on the lip has paresthesia[3].

FLOOR OF THE MOUTH- The early lesion is characterised by redness, mild elevation, weakly delineated boundaries, and minimal induration. As the tumour expands, its edges become defined, raised, and rolling, while the centre develops ulceration and induration.

There is a background of erythroplakia and leukoplakia visible.

Large lesion bulge into submental space and grow into the soft tissues of the neck through myelohyoid muscles. After the removal of anterior teeth, certain cancers invading the mandible could be seen and through it tumour is spread to the lip and gingival sulcus[3].

TONGUE- Most frequent complaint is mild irritation. As the ulceration progresses, the pain is felt in the external auditory canal and becomes increasingly severe. Speech and deglutition is affected due to extensive infiltration of the muscles. It is also associated with foul and necrotic odour.

Eventually, the tongue becomes fixed in one position and loses its ability to extend to the unaffected side.

Behind the mylohyoid muscles is the posterior oral tongue lesions, and they could present as a lump near the angle of mandible of the neck. Invasion to hypoglossal nerve is rarely seen[3].

BUCCAL MUCOSA-

Small lesions often cause a "lump" sensation on the tongue. Mild pain is experienced if the lesion does not spread to the posterior lingual and dental nerves. The ear is often mentioned as a possible referral point for pain. Parotid enlargement is seen if there is obstruction to Stenson duct. It also causes trismus if there is involvement of buccinator and masseter muscles[3].

RETROMOLAR TRIGONE, HARD PALATE AND GINGIVA

Ill-fitting dentures, loose teeth, pain, non-healing sore throat and other similar issues are all possible manifestations.

History of inappropriate root canal therapy and dental extractions is common.

Lower lip paresthesia occurs due to involvement of inferior alveolar nerve which is affected by the tumors invading the mandible. Evidence of leukoplakia pre-exists.

Lesions of the retromolar trigone cause pain in the external auditory canal and the preauricular region. Trismus developed due to involvement of pterygoid muscles.

Submucosal mass and dental symptoms are seen in intra-alveolar SCC and X ray shows lytic lesion in the mandible.

Minor salivary glands may enlarge slowly and can cause a submucosal tumour to form and eventually ulcerate at their centre[3].

Clinical features of HPV- induced Oral squamous cell carcinoma

HPV-positive patients who have head and neck squamous cell carcinoma are more likely to be younger, to have never smoked, and to use alcohol just socially. Patients with HPV positive HNSCC patients may engage in oral sexual behaviour or have several sex partners. Survivability is better among patients with HPV-positive HNSCC than those with HPV-negative HNSCC. Oropharynx and base of tongue are typical sites for the development of HPV-induced squamous cell carcinoma[61-66].The tonsillar crypts are encased in reticulated epithelium and are located surrounding the immune system in close proximity. HPV infection and subsequent malignant transformation may compromise the immune system[65].

The HPV-induced squamous cell carcinoma cannot be distinguished from non-HPV-induced squamous cell carcinoma by any particular histologic features. Identification of HPV-positive and -negative patients is essential for administering targeted therapy. Understanding the pathophysiology of HNSCC in all people is essential for identifying the unique targets of treatment. The prototypical HNSCC is very slightly distinguishable from the physical characteristics of HPV-driven carcinogenesis. SCCs caused by HPV are primarily non-keratinizing SCC. They are sometimes referred to as poorly differentiated carcinomas or basaloid carcinomas due to the lobular growth of cells with hyperchromatic nuclei, minimal cytoplasm, and apparent mitotic activity[67-71].

The clinical manifestation of tumour and neck stage in HPV-positive HNSCC is unique.

In contrast to non-HPV-related carcinomas, which are typically found at a later N-category stage, carcinomas caused by HPV are identified at a more treatable stage I (T-category stage)[71].

The TNM staging of the Oral squamous cell carcinoma[72]

TNM cancer staging is an evaluative system that uses the primary tumour's size (T), involvement of local lymph node (N), and distant metastases (M) to determine the amount of tumour progression throughout the body. This classification is crucial for the planning of the therapeutic course, calculating the likelihood of recurrence, and gauging overall survival. The disease's anatomic spread is the only prognostic factor taken into account in this classification; other prognostic criteria, including comorbidities or treatment, are not.

Carcinoma of the oral cavity is staged according to the TNM system. Squamous cell carcinoma makes up the great majority of cases that apply, however various cancers of the epithelium and minor salivary glands are included as well. The material that follows is based on the eighth edition of the American Joint Committee on Cancer (AJCC), which was released in 2017 and updated in 2018.

Table 4: TNM staging of OSCC

PRIMARY TUMOUR (T)	
TNM STAGING	**EXTENT OF PRIMARY TUMOUR**
TX	Primary tumour undetermined
Tis	Tumour in situ
T1	Tumour is 2 cm (largest dimension) and its depth of invasion (DOI) is 5 mm
T2	
i.	Tumour lesser than or equal to 2 cm in diameter and between 5 to 10 mm of DOI
ii.	Tumour more than 2 cm but lesser than 4cm in diameter with a DOI of 5 mm to 10 mm.
T3	
i.	Tumours with DOI of more than 10 mm or
ii.	Tumours larger than 4cm in diameter with a DOI of 10 mm
T4	Moderately advanced or Very advanced
T4a	Moderately advanced- local disease
i.	Tumour larger than 4 cm with more than or greater than 10mm DiiOI
ii.	Tumour has spread to nearby organs or tissues (through the maxillary sinus, mandibular or maxillary cortical bone, into facial skin etc
T4b	Extremely advanced- local disease

iii.	When the tumour has spread to the pterygoid plates or the base of the skull, masticator space and invasion to the internal carotid artery
REGIONAL LYMPH NODE (N)	
NX	Regional lymph node cannot be assesed
N0	No metastatic regional lymph node
N1	Metastases of single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastases are specified as N2a, N2b and N2c
i. N2a	Metastasis in a single ipsilateral lymph node, $> 3\ cm$ but $\leq 6\ cm$
ii. N2b	Multiple ipsilateral lymph node metastasis with none > 6 cm
iii.N2c	Bilateral or contralateral lymph node metastases with not > 6 cm in greatest dimension
N3	Lymph node metastasis with > 6 cm in maximum dimension
DISTANT METASTASES (M)	
cM0	No signs of metastases
cM1	Distal metastases
pM1	Microscopically verified distal metastasis

NOTE- To categorise as T4 requires more than just superficial erosion of the bone or tooth socket caused by gingival primary.

STAGE GROUPS[72]

Groups of patients are given a prognosis based on their tumor's stage. It is categorised similarly to how other head and neck cancers are:

Table 5: Stage Grouping of OSCC

STAGE GROUP	TNM STAGING
STAGE 0	Tis, N0, M0
STAGE I	T1, N0, M0
STAGE II	T2, N0, M0
STAGE III	T3, N0, M0 or {T1, T2, T3}, N1, M0
STAGE IVA	T4a, {N0, N1}, M0 or {T1, T2, T3, T4a}, N2, M0
STAGE IVB	{Any T}, N3, M0 or T4b, {Any N}, M0
STAGE IVC	{Any T}, {Any N}, M1

Histopathological grading of OSCC

In the OSCC histopathological grade for lip squamous cell carcinoma, Broder's pioneered the use of histopathological grading. It was predicated on the variances in tumour differentiation. Anneroth et al., Bryne et al. and Jakobsson et al. suggested more intricate grading schemes. Later, characteristics of the tumour itself, like differentiation, as well as those of the tumour-host interface, like invasion patterns and host responses were all considered by these multifactorial systems. They influenced the establishment of the concept of cohesive, dyscohesive, and non-cohesive patterns of invasion in the minimal data sets of the Royal College of Pathologists (United Kingdom), to which a dispersed pattern was later added. A novel

feature that should be added to these systems is the ability to assess myofibroblasts, or cancer-associated fibroblasts, using alpha smooth muscle actin immunohistochemistry. However, the World Health Organization (WHO) did not support such multifactorial systems and instead focused on bare-bones data sets and uniform histopathology reporting. According to the degree of keratinization and nuclear pleomorphism, tumours of the head and neck, according to the most recent edition of the Classification of Head and Neck Cancers only recognises three different levels of differentiation for traditional OSCCs: well, moderately, and poorly-differentiated[73,74].

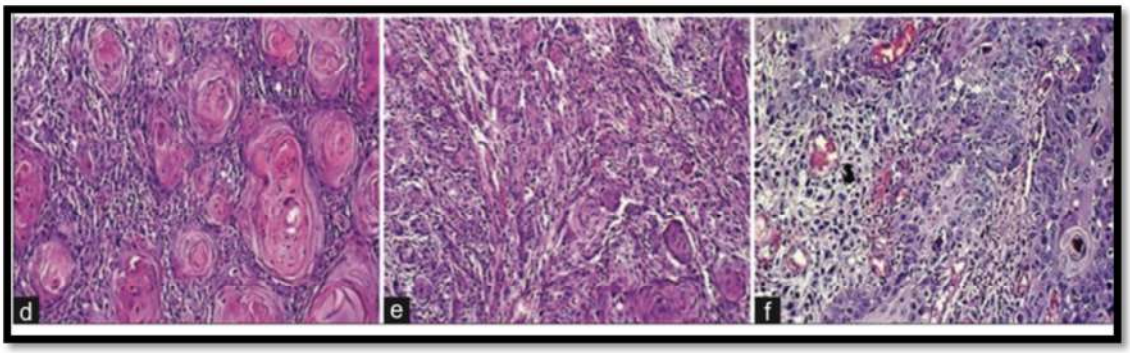


Figure 11- Histopathological grading of OSCC as per degree of differentiation, Low power, H&E: (d) Well (e) Moderately and (f) Poorly differentiated

(Image sourced from Estimation of serum sialic acid in oral submucous fibrosis and oral squamous cell cancer by Chittemsetti S, Manchikatla PK, and Guttikonda V. J Oral Maxillofac Pathol 2019;23:156–160)

IMMUNOHISTOCHEMISTRY-

p16 ImmunoHistoChemistry (IHC)

p16 (or p16INK4a, CDKN2A, cyclin-dependent kinase inhibitor 2, and multiple tumour suppressor 1) is a protein with various names. It acts as a tumour

suppressor by preventing cell division and prolonging the G1 to S phase transition. Overexpression of p16INK4a protein, a cyclin-dependent kinase inhibitor, is a promising surrogate biomarker. It serves as the foundation for the gold standard test for detecting HPV involvement in tumours. During the process of host cell immortalization, we observed the overexpression of the tumor-suppressor gene p16.Downregulation of Rb gene liberates E2F factors in response to high-risk HPV E7. In the presence of free E2F, p16INK4a is induced or upregulated. p16 is now universally accepted as the standard test in clinical evaluation because of its quick, inexpensive method of detection. However, it's important to keep in mind that not all tumours with a p16 positive status are also HPV positive. As much as 20% of p16-positive HNSCCs have not been linked to HPV. A small number of studies have found that patients with positive results for HPV DNA and mRNA do not have abnormally high expression of p16. According to another study, patients with OPSCC who tested positive for p16 but negative for HPV antibodies had a dismal prognosis[75-78]. According to these results, p16 overexpression is insufficient by itself to accurately detect HPV infection in HNSCC. However, p16 IHC is the only option utilised by many doctors because it is reasonably priced, has clear criteria for interpreting stains, and has been thoroughly examined. Along with p16 INK4a IHC, DNA and RNA based technologies will be required in the future for HPV identification.

Other immunohistochemical markers that shows positive to tumour cells includes CK8, CK19, CK5/6 and often p53. Additional factors in determining OSCC outcomes include expression of the markers p53, EGFR, and cyclin A[79,80].

TREATMENT MODALITIES FOR OSCC-

LIPS-

Surgery as well as Radiotherapy (RT) has equal cure rate in early lesions. Surgery is the recommended treatment for lower lip lesions diameters up to 2 cm outside the commissure. But RT is given as an option if the commissure is affected, including upper lip cancers or upper lip lesions longer than 2 cm.

Combining treatment modalities is sometimes necessary for more advanced lesions. For high-grade, stage-advanced, or recurrent lesions in the neck, an elective treatment option may be considered.

Other forms of treatment include interstitial brachytherapy, external beam radiation therapy (EBRT), and combinations of both[3].

FLOOR OF THE MOUTH-

Both surgical excision and radiotherapy (RT) are successful for T1 and T2 tumours. Necrosis of bone, soft tissue, and dry mouth are a considerable risk after radiotherapy, hence most patients choose for surgical treatment instead. If there is a tiny lesion of TX it is preferred to treat with re-excision or brachytherapy.

Recommendations for treatment in moderately advanced instances include rim excision or segmental mandibulectomy followed by osteomyocutaneous free flap repair and common radiation therapy (CRT) or post op radiation therapy is added as per pathologic findings. Treatment of midline tumours with a clinical N0 neck is accomplished through bilateral functional neck dissection.

Coupling surgical intervention with subsequent CRT is recommended in more advanced patients but they have a poor prognosis. Clinical circumstances will dictate whether primary CRT or RT are considered in addition to palliative care[3].

Surgical treatment includes-

1. Wide Local Excision- Lesions with 5mm or less in size. Involvement of the submandibular gland and duct necessitates the removal of both in a single operation.

2. Marginal Mandibulectomy- Marginal mandibular resection is the removal of the mandible's rim in tandem with the removal of the underlying primary lesion. They may combine with post-operative RT

3. A segmental mandibulectomy involves removing the floor of the mouth that has invaded the cortical bone and reconstructing it with an osteomyocutaneous free flap[3].

Other treatment includes:

Irradiation technique- Brachytherapy or Intraoral Cone RT is used to treat patients with superficial T1 cancer.

External beam radiation therapy (EBRT)- Cancers affecting the anterior floor of the oral (or palate) including both neck nodes level I and II, as well as the full width of the mandibular arch.

Interstitial irradiation- T1 and T2 lesion minimally extending to tongue mucosa and restricted to the floor of the mouth.

Intraoral Cone Irradiation- Used in well circumscribed anterior superficial lesions[3].

ORAL TONGUE-

In early lesions, the success rate of surgery and radiotherapy (RT) is comparable, and for more advanced tumours, a combination of the two is necessary. Tiny lesion (TX) removed with excisional biopsy. Partial glossectomy with primary closure performed trans orally is the recommended treatment for early lesions (T1 or T2). If the invasion is deeper than 4 mm, a neck dissection may be necessary for treatment. Glossectomy, neck dissection, tongue reconstructions, and post-operative RT/CRT are the recommended treatments for moderately advanced cases (T2 or T3). Excellent functional outcomes and quality of life can be achieved by intensive treatment of advanced lesions (T4)[3].

Surgical treatment includes-

1. Partial glossectomy and primary closure for early lesions. Extensive resection and free flap repair can be needed for deeper lesions.
2. Partial glossectomy followed by primary closure, skin grafting, and flap restoration is the standard treatment for a lesion that has progressed to a moderate stage.
3. Glossectomy (near total or entire) and even laryngectomy (total) are sometimes necessary for patients with T4 lesions, which are considered advanced.

Irradiation, either through intraoral cone placement or interstitial RT, is another option. Post-operative radiation therapy (RT) or concurrent radiotherapy (CRT) to the primary site is often part of these combined treatment plans[3].

BUCCAL MUCOSA-

Primary closure after excision is possible for benign lesions smaller than 1 cm in size. The standard course of treatment is surgical for lesions that are two to three centimetres in length. RT is used to treat small lesions on the lip commissure.

Surgical treatment includes bone resection if mandible and maxilla is invaded by the tumor lesion with free flaps to repair a soft tissue or bone defect. Bilobed free flaps are used to reconstruct the full thickness of the cheeks to get the desired cosmetic results.

Other treatment includes- Electron beam irradiation, intraoral cone irradiation, and interstitial irradiation are all used to protect the normal tissues on the opposite side of the body[3].

RETROMOLAR TRIGONE, HARD PALATE AND GINGIVA

In the case involving the lower alveolar ridge, surgical treatment alone or RT or CRT administered after surgery is the method of choice. Segmental mandibular excision with free flap repair is recommended for more advanced or severe patients. Ameloblastoma- surgery is the gold standard, however there is a chance of local recurrence. It is recommended to operate on retromolar trigone lesions if they are small and diagnosed at an early stage. A combination of surgery and post-operative RT or CRT is used to treat advanced carcinomas, while RT is the treatment of choice for superficial lesions covering a large region. Resection of the hard palate and upper alveolar ridge, either alone or in combination with CRT or RT, is the treatment of choice.

Other treatment includes- Irradiation technique by intraoral cone for small lesions. The ipsilateral mixed beam or intensity modulated radiation therapy (IMRT) is the preferred method of treatment for well lateralized lesions[3].

Prognosis of HPV induced OSCC

Patients with HPV-positive HNSCC, especially those with oropharyngeal carcinoma, who undergo radiotherapy, chemotherapy, surgery, or a combination of these have a better prognosis than those with HPV-negative HNSCC who undergo the same treatments. All of the aforementioned data are from single-centre retrospective case series research from recent years. According to the studies, there is a lower probability of disease failure in individuals with HPV positive squamous cell carcinoma compared to people without the virus. The prognosis for HPV-positive HNSCC is better than that for HPV-negative HNSCC, however the explanation for this is unclear. Factors that increase the likelihood of a favourable outcome for HPV-positive cases include an earlier age at diagnosis, reduced usage of alcohol and cigarettes, a more aggressive treatment strategy, a lower risk of recurrent tumours, and a unique disease biology[81-84]. Having a wild-type version of TP53, which allows cancer cells to have an apoptotic response to radiation and chemotherapy, may contribute to the favourable outcome of HPV-induced SCC[85].

Use of tobacco products may change the clinical course of HPV-positive HNSCC, which could have a negative impact on the prognosis. The tumour suppressor signaling pathways are targeted by the high risk HPV E6 and E7 oncoproteins. In transcriptionally active HPV infections, HPV16 E6 has the key transformative property of inducing p53 perversion via the ubiquitin route, while the key transformative property of HPV16 E7 is inactivating pRb. Increased expression of

CDKN2A, which encodes p16INK4a, is linked to this occurrence. Without regard to the presence of an HPV infection, p16INK4a positive and the absence of TP53 gene alterations are both strongly related with improved overall survival[86].

Using a three-pronged approach, researchers at the University of Foggia in Italy were able to identify HPV in oral malignancies. The research was undertaken in the Department of Surgical Sciences' Section of Anatomic Pathology and Cytopathology. All the head and neck malignancies tested positive for HPV by polymerase chain reaction and in situ hybridization, and were also positive for HPV by immunohistochemistry for p16. Furthermore, when compared to HPV DNA PCR and in situ hybridization, p16 IHC demonstrated the best level of sensitivity as a single test. Oropharyngeal squamous cell carcinoma (OPSCC) and oral cavity squamous cell carcinoma (OSCC) were reported to have a sensitivity of 100% and a specificity of 73% and 93%, respectively. However, they did not study the association of HPV with the histological grading using p16 IHC[87].

Oral and pharyngeal malignancies have been linked to HPV, according to a meta-analysis conducted by Nallan C.S.K. et al. utilising PubMed (1995–2015), Medline, Cochrane, Science Direct, and an internet search. After compiling the data, they discovered that 588 out of 1497 individuals had been given a positive HPV diagnosis by one of several methods. Using the odds ratio and 95% confidence interval, they found that the risk of HPV was significantly higher in the case groups compared to the controls. However, no systematic review analysis of the correlation between HPV infection and p16 histological grading was conducted in their work[88].

A study conducted at Bangalore in 2014 shows that 26/30 (86.66%) OSCC cases showed p16 positive representing HPV infectivity and found a strong

association between HPV infection and OSCC. They also found a diffuse pattern (grade3) of p16 expression in a poorly differentiated OSCC and opined that p16 correlates with aggressiveness of the OSCC[2].

In another 2017 prospective study conducted in Kanpur, 50 cases of oral cancer suspicion were chosen at random for HPV identification using HPV DNA (PCR) and p16 IHC technique. HPV DNA PCR found only 12% of cases, with only 1/17 (5.8%) cases of oral dysplasia and 5/33 (15.1%) cases of oral carcinoma, while p16 expression was seen in 62% of cases (31/50). In addition, 83.3% of the HPV DNA-positive patients were found to have a positive p16 IHC stain. P16 positivity was also observed to be higher in oral carcinomas than in oral dysplasia, within the context of histological subtypes[4].

Prakash P. et al. examined 69 SCC lesions and 21 cases of leukoplakia with or without dysplasia[89]. Diffuse nuclear and cytoplasmic staining was reported to be the most common p16INK4a expression pattern in the majority (71%) of oral SCC patients. A total of 57.1% of leukoplakia cases tested positive for p16INK4a overexpression. Overexpression in 69 cases of oral cancer showed a diffuse pattern in 31.9%, sporadic in 24.6%, and focal in 14.5%. They examined p16INK4a expression in 21 cases of leukoplakia and found that 23.8% of cases displayed diffuse expression, 9.5% displayed sporadic expression, and 4.5% displayed focal expression. According to their research, the tongue is the most prevalent location for OSCC to manifest.

In a study done in the Dharwad district of North Karnataka, Oral submucous fibrosis (OSF) lesions were found to be p16 positive in 83.3% of cases displaying 0-30% tumour cells positivity, and in 16.7% of patients showing 61-90% tumour cells positivity[90]. p16-positive tumour cells were observed to be more prevalent in OSCC

with OSF (31%-60%) than in OSCC without OSF (53.3%). Additionally, they also found p16 positivity ranged from 61-90% in 40% OSCC with OSF and 46.7% without OSF. Moreover, the percentage of tumour cells that are positive in OSCC with or without OSF was shown to be higher than in only OSF lesion in their investigation. p16 overexpression was either observed in the nucleus, the cytoplasm, or both. The measured level of intensity was either mild, moderate, or intense.

Hashmi A et al. investigated 144 cases of head and neck squamous cell carcinoma[91]. They found that 22.9% had overexpressed p16, while 21.5% were focally positive and 55.6% were negative. Based on the percentage of p16 expression, they found 4.9% cases with p16 expression at or above 70%, 9.0% with expression between 51% and 70%, 9.0% (with expression between 11% and 50%, and 77.1% with no expression or expression of less than 10%.As a comparison, they used tonsils and cervical cancer. The possibility of cytoplasmic staining was also taken into account with nuclear staining. There was a four-point scale used to rate the intensity of the staining: none (zero), weak (1+), patchy/moderate (2+), and diffuse/high (3+). Conversely, the proportion of cancer cells that were positively stained was determined as a continuous variable. Focal positivity was defined as intermediate to strong staining in fewer than 10 percent of cancer cells, while positive was defined as intermediate to strong staining in more than 10 percent of cancer cells. p16 immunostaining results were also divided into subsets based on the percentage of positive cells.

Ralli et al. conducted a prospective research in which 75 HNSCC patients were included[92]. From a total of 75 patients, 78.7% showed positive for p16 (across all grades), while only 21.3% showed negative for p16. They observed expression of

p16 was positively correlated with the paan chewing habit and was higher in people who did not smoke or drink alcohol. Also, their study found a strong correlation between p16 expression with both the number of sexual partners and the tumour's histological grade, as well as lymph node metastasis but had no significant correlation with a history of aberrant sexual practises.

Nuclear versus cytoplasmic positive was used to categorise p16 IHC expression. Biopsies were considered positive if they contained more than 5% positively stained cells (the "cutoff") and negative if they contained less than 5% positively stained nuclei and cytoplasm. They defined sporadic positivity as 5-10% of labelled nuclei and cytoplasm displaying weak and dispersed positivity, focal positivity as 10-30% of labelled nuclei and cytoplasm displaying strong positivity and spreading in one tissue area, and diffuse positivity as 30-85% of labelled cells displaying strong positivity and spreading diffusely across multiple tissues. It was found that biopsies that were stained all over (Grade III) had high levels of p16. Grade II expression was regarded to be present when there were clusters of it, while Grade I expression was found only in isolated spots (Grade I).

Zeyi Deng and his colleagues analysed 150 tumour samples from the nasopharynx, oropharynx, hypopharynx, larynx, and from the oral cavity[93]. Overexpression was defined as greater than 40% p16INK4a-positive cells, and expression was evaluated on a scale from 0 to 4. When strong nuclear and/or cytoplasmic reactivity was seen, cases were labelled as p16INK4a-positive. Based on previous scoring systems, they set the following criteria for p16INK4a immunoreactivity (p16INK4a expression) in their investigation: a score of 0 (no staining), score of 1 (positive in 1–10% of tumour cells), a score of 2 (12–40%

positive), a score of 3 (50–70% positive), and a score of 4 (>70%). A score of 3 or 4 is considered to be indicative of p16INK4a overexpression. They also observed that patients with OPSCC who overexpressed p16INK4a had disease-free survival for three years compared to those who did not.

Lewis et al. study found that out of 239 patients, 187 tested positive for p16[94]. When tested with ISH for HPV, 139 of them were positive, or 74%. Out of the remaining 48 cases, 45 had sufficient samples for polymerase chain reaction analysis. Only 19 of the SCCs tested positive for HPV, in a total of 26 SCCs that tested positive for p16 but negative for HPV. Each experiment had a positive control consisting of a previously identified p16- positive head and neck SCC patient and a negative control consisting of portions of normal tonsil. Nuclear and cytoplasmic staining was seen, and its intensity was quantified using a quartile scale by a single investigator. Negative (a score of 0), positive (1–25%), intermediate (26–75%), high (76–100%), and superlative (100–100%). On the other hand, cases were classified into either positive (1+ to 4+) or negative (0+) categories for the purposes of analysis. Referenced in said study the prognosis for p16 positive, HPV negative oropharyngeal SCC is comparable to that of p16 positive, HPV positive tumours. Moreover, it is markedly better when compared to p16 negative cancers. Their results supported that p16 immunohistochemistry can be used on its own to classify the risk of oropharyngeal SCC.

According to the Ferreira study, p16 positivity was shown to be prevalent in 32.14 percent of oropharyngeal carcinomas[95]. Researchers have observed that the incidence of oropharyngeal cancer caused by HPV is quite low in studies involving participants from developing countries.

Of the total 252 patients who took part in the trial, 87.7% were men. A total of 81 cases (32.14%) were positive for p16, while 171 cases (68.15%) were negative. Patients with p16 positive group were younger (50-59 years old), more educated, in an earlier stage of disease, and non-smokers or non-drinkers. Strong and diffuse staining of more than 75% of nuclei and cytoplasm was utilised as a cut off for determining positive p16 expression.

According to the Azizi SA et al. study, nuclear and cytoplasmic staining of tumour cells constitutes positive immunohistochemistry expression of p16[96]. When over than 10% of tumour cells show moderate to strong staining, the tumour was considered positive. Almost 93% of OSCCs revealed p16 expression in their study, and 50% of those cases displayed high levels of expression. Protein expression levels were graded as either not stained at all (0), very faint or weak staining (1), moderate staining (2), or very high/strong staining (3).

According to the findings of a study carried out by Pandey P et al., p16 positivity was found in 60% of cases[97]. Immunohistochemical analysis of p16 expression was performed according to nuclear and cytoplasmic positive states. A result was considered positive over the cut off of 5% positively stained cells. Biopsies with a diffuse pattern was defined as more than 30%–85% of labelled cells exhibiting strong positive and spreading throughout several tissue regions and were found to have significant p16 IHC expression (Grade III). Moderate expression (Grade II) was defined as widespread positivity with greater than 10%–30% of labelled nuclei and cytoplasm, highly positive and spreading in one tissue area, whereas low expression (grade I) was defined as sporadic positivity with 5%–10% of labelled nuclei and cytoplasm, weakly positive and dispersed throughout the tissue. Research into p16

expression may help doctors make more informed decisions about tumour aggressiveness, treatment options, and even the viability of a vaccination programme for people at high risk. Hence, p16 immunohistochemical expression can be used as HPV surrogate marker. Moreover, they discovered a direct and statistically significant relationship between p16 and age, tumour location, abnormal sexual behaviours, and lymph node involvement. However, their study did not show any correlation between p16 expression and histological grading.

The research of Meng et al. found overexpression of p16 in 81 (5.51%) of the 1470 patients while 78 (5.31%) of the 1470 cases, HPV positivity was detected[98]. Nuclear and cytoplasmic positivity were determined to be positive reactions for the purpose of estimating p16 [INK4a] expression and were rated semi-quantitatively in accordance with established criteria: The scores ranged from 0 (no positive cells) to 1-4% of positive cells were sporadic, 5-79% of positive cells were focal and more than or equal to 80% of positive cells received a score of diffuse. Both nuclear and cytoplasmic staining were required for p16-positive cells to meet their criteria with at least 80% of tumour cells p16-positive. Immunohistochemistry detected p16 overexpression in 5.51 percent of OPSCC samples, and PCR testing for HPV found positivity in 5.31 percent, suggesting a strong association between the presence of HPV and p16 overexpression. Eighty-nine percent of patients whose tumours expressed p16 were smokers, and about 70% of patients with p16 expression also reported having used alcohol in the past in their study. Also, patients who express p16 are more likely to respond well to surgery and radiation therapy, and the authors' research shows that this is true even for patients with early-stage primary OPSCCs.

MATERIALS AND METHODS

Study design: Cross sectional study

Study population and data collection: All the specimens of oral carcinoma received at the Histopathology laboratory at KLE'S DR. PRABHAKAR KORE CHARITABLE HOSPITAL and MEDICAL RESEARCH CENTER, BELAGAVI from January 2021 to December 2021

Sample size: A total of 50 oral carcinoma cases.

Selection criteria:

Inclusion criteria: All the specimens diagnosed histopathologically as oral carcinoma, including biopsies.

Exclusion criteria:

1. All poorly preserved and inadequate specimens.
2. Cases diagnosed as with premalignant lesions or dysplasia

Ethical clearance: The ethical clearance was acquired from Institutional Ethics Committee, JNMC, Belagavi prior to the commencement of study.

Method of data collection

Procedure: All the biopsies and whole specimens of oral carcinoma received at the histopathology laboratory were collected, numbered and kept for fixation in 10 % formalin overnight. The specimens were taken the next day for grossing and representative sections of tissue were given in different capsules. These capsules were

taken for processing in the tissue processor. The tissues in the capsules underwent the process of dehydration in upgraded alcohol solutions, clearing in xylene and impregnation with paraffin wax in the tissue processor.

The tissues were then taken from the capsule and embedded in molten wax for block preparation. Sections measuring 3-4 microns each were cut using microtome and taken on to the slides. 50 slides were stained using hematoxylin and eosin stain (H &E) for the histological grading and histopathological evaluation. The slides for IHC were pre-coated using Poly-L-Lysine and stained for IHC using specific mouse monoclonal antibody to p16. For positive control, Carcinoma Cervix was taken and for negative control, IHC staining was done without the use of primary antibody. After dipping slides in xylene, they were mounted with a coverslip using Dibutylphthalate Polystyrene Xylene (DPX).

Both H & E and p16 IHC staining procedures were performed and mounted.

STEPS OF p16 IHC PROCEDURE

(TRIS buffer +EDTA)- Buffer solution required amount of buffer is prepared

Tissue section is cut on a microtome with 3 microns thickness and collected on coated slides

↓

Bake the sections at 37 degree celsius overnight. Before test bake it at 60 degree celsius for 1 hour

↓

Deparaffinise steps-
Xylene I- 10 minutes
Xylene II- 10 minutes
Absolute alcohol I- 10 minutes
Absolute alcohol II- 10 minutes
Rinse in water- 5 minutes
Rinse in distilled water- 1 minute

↓

Antigen retrieval (TRIS buffer +EDTA)- Buffer solution

↓

Required amount of buffer is prepared and cook the slides in pressure cooker for 3 whistles

↓

Allow it to cool to room temperature for 15 minutes

↓

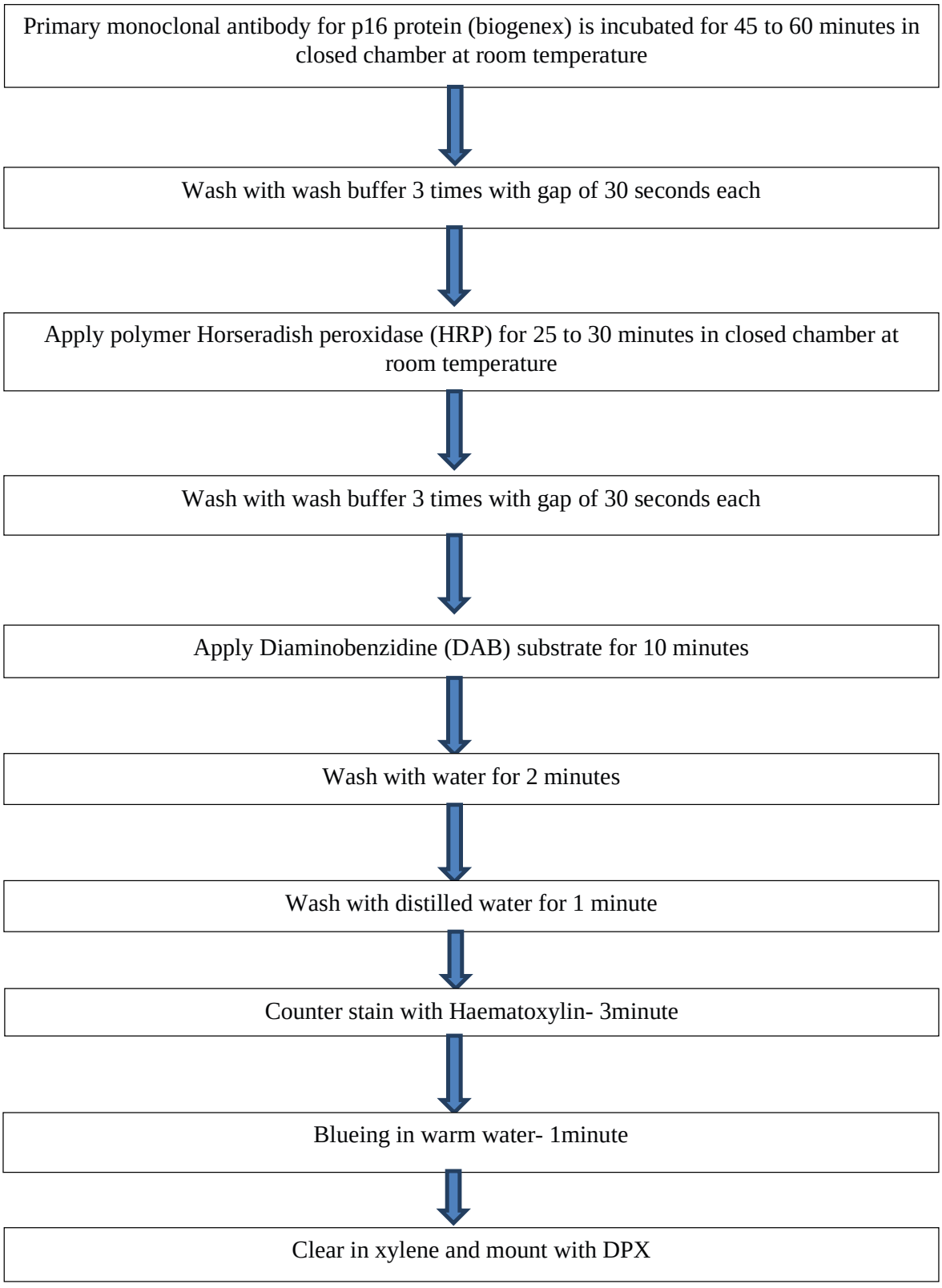
Wash with wash buffer 2 times with gap of 30 seconds each

↓

Wash with water buffer 3 times with a gap of 30 seconds.

↓

Apply 3% hydrogen peroxide- 8 to 10 minutes


Primary monoclonal antibody for p16 protein (biogenex) is incubated for 45 to 60 minutes in closed chamber at room temperature
Wash with wash buffer 3 times with gap of 30 seconds each
Apply polymer Horseradish peroxidase (HRP) for 25 to 30 minutes in closed chamber at room temperature
Wash with wash buffer 3 times with gap of 30 seconds each
Apply Diaminobenzidine (DAB) substrate for 10 minutes
Wash with water for 2 minutes
Wash with distilled water for 1 minute
Counter stain with Haematoxylin- 3minute
Blueing in warm water- 1minute
Clear in xylene and mount with DPX

All the slides were examined and reported using MODIFIED BRODER'S GRADING SYSTEM and graded as per degree of differentiation by a pathologist on H & E staining as:-

1. Well differentiated (Grade1)
2. Moderately differentiated (Grade 2)
3. Poorly differentiated (Grade3)

The p16 IHC slides were assessed under Olympus BX41 microscope. Selected pictures were taken using JENOPTIK SUBRA digital camera using the GRYPHAX software.

CRITERIA USED TO INTERPRET p16 OVEREXPRESSION INCLUDES[94]

1. Pattern of staining
   i. Both nuclear and cytoplasmic staining as Positive
   ii. Only cytoplasmic staining and complete absence of staining as Negative
2. Percentage of tumor cells staining
   i. 1-25% - 1+
   ii. 26-50% - 2+
   iii. 50-75% - 3+
   iv. >75% - 4+

3.	Intensity of tumor cells staining	Score
❖	No tumor cells stained -	0
❖	Mild/Weak/ Bare (singly dispersed cells) -	1
❖	Moderate/Patchy -	2
❖	Strong/Diffuse -	3

For evaluation of p16 immunohistochemical staining, the evaluation was done using the 400x magnification of the microscope. All the cases showing a visible brown staining for nucleus and cytoplasm was taken as positive for p16 marker.

Appropriate scoring was done for the percentage of tumour cells and the staining intensity of tumour cells showing positive nuclear and cytoplasmic staining for p16 marker will be considered as follows:

- ❖ Staining Score 0: No tumor cells stained
- ❖ Staining Score 1: Mild/Weak/Bare singly dispersed cells
- ❖ Staining Score 2: Moderate/Patchy
- ❖ Staining Score 3: Strong/Diffuse

Statistical analysis

Data obtained was entered in Microsoft Excel software and analyzed and expressed in percentages and proportions. Cases showing p16 positivity and its association with histopathological grading were studied. A p-value of less than 0.05 was considered statistically significant.

RESULTS

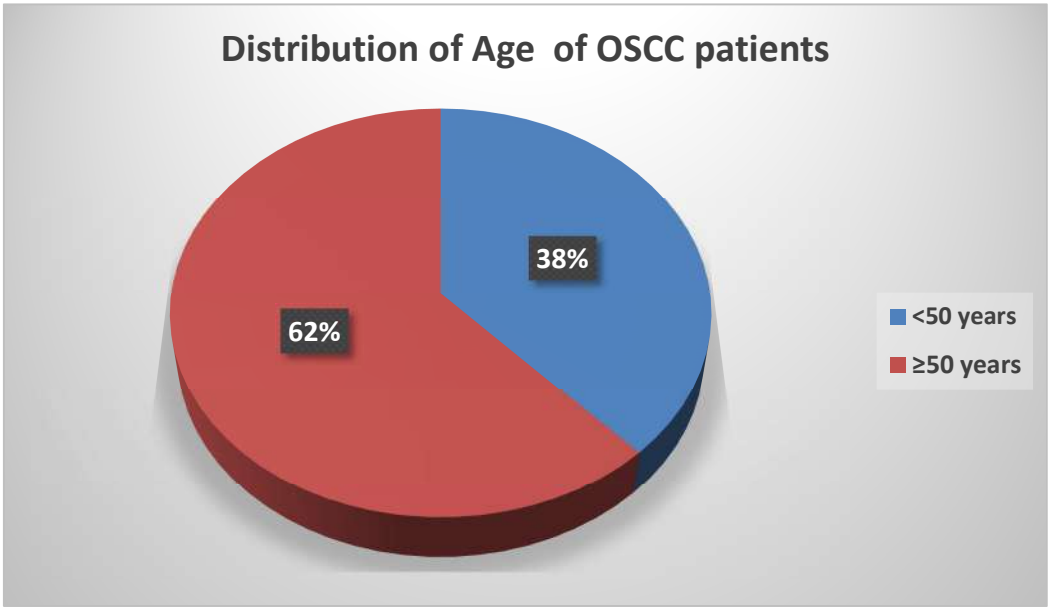
In the present study, a total of 50 cases of OSCC (oral squamous cell carcinoma) were studied. All the cases were evaluated for H&E stain for histological grading followed by the assessment of p16 expression. Cases showing p16 positivity and its association with histopathological grading were studied.

Data of 50 cases of OSCC were analysed. In these 50 cases, majority (34) were resected specimen and 16 were biopsy specimen.

Table 6: Age distribution of patients with OSCC

Age groups (years)	Frequency	Percentage (%)
<50	19	38
≥50	31	62
Total	**50**	**100**

Figure 12. Distribution of Age in patients with OSCC

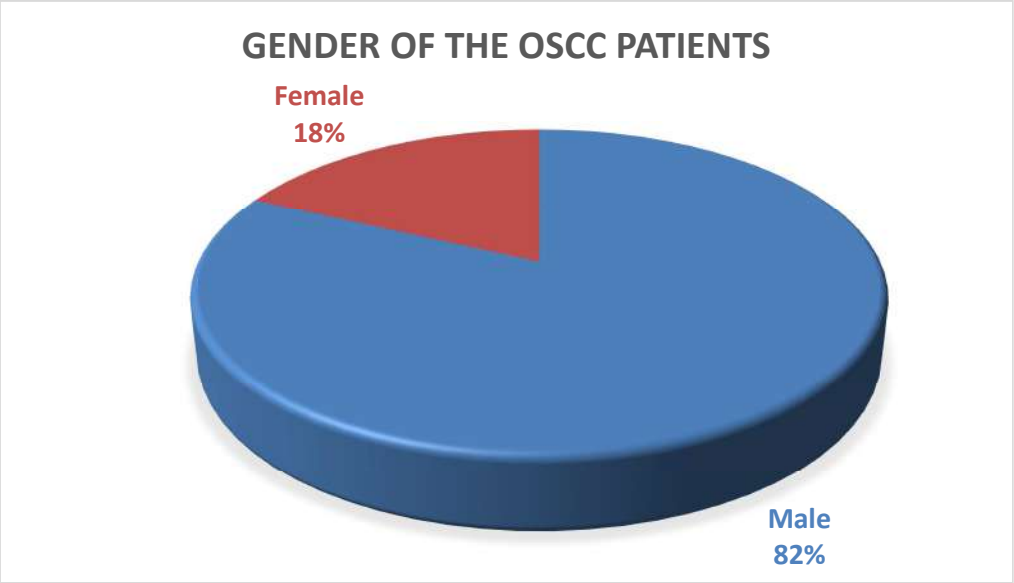


In this study age of the patients ranged from 31 to 70 years with a mean age of **53.78±11.76 years.** 62% cases are more than 50 years and 38 % less than 50 years of age. 82% were male and 18 % female with a sex ratio of 4.5:1.

Table 7: Sex of the patients with OSCC.

Sex	Frequency	Percentage (%)
Male	41	82
Female	9	18
Total	**50**	**100**

Figure 13. Sex of the patients with OSCC.

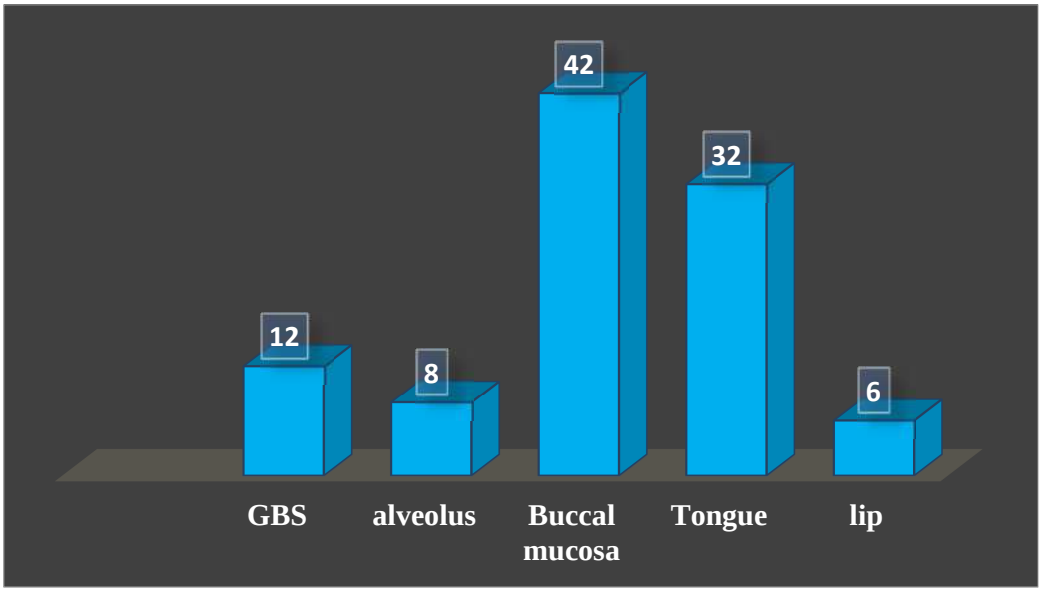


In the sex distribution of the OSCC cases 82% were male patients and 18 % were female with a sex ratio of 4.5:1.

Table 8: Site of involvement of Oral Squamous Cell Carcinoma.

Site of lesion	Number (n)	Percentage (%)
Alveolus	4	8
Gingivo buccal sulcus (GBS)	6	12
Buccal mucoca	21	42
Tongue	16	32
Lip	3	6
Total	**50**	**100**

Figure 14. Site of involvement of Oral Squamous Cell Carcinoma.

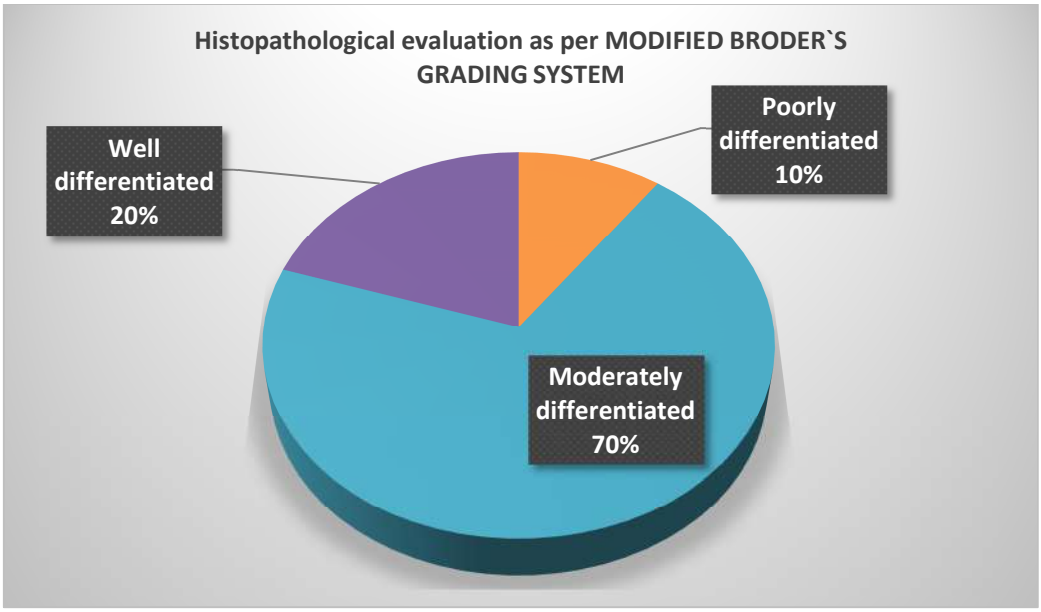


The most common site of involvement was observed in buccal mucosa (42%) followed by the tongue (32%) and gingivobuccal sulcus (GBS-12%).

Table 9: Histopathological evaluation as per MODIFIED BRODER`S GRADING SYSTEM.

Histopathological diagnosis	Frequency	Percentage (%)
Poorly differentiated	5	10
Moderately differentiated	35	70
Well differentiated	10	20
Total	**50**	**100**

Figure 15. Histopathological evaluation as per MODIFIED BRODER`S GRADING SYSTEM.

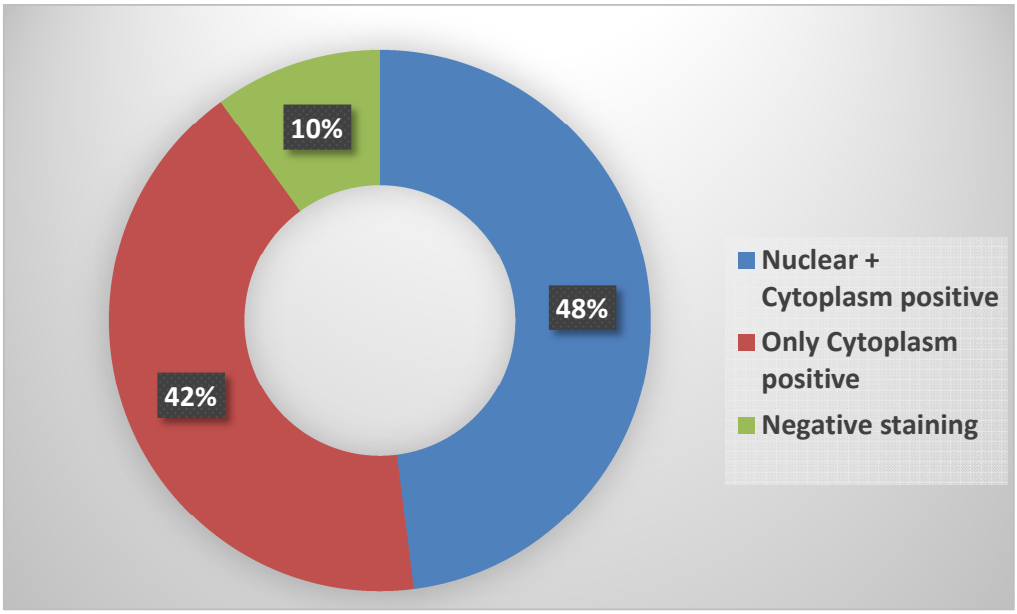


Out of 50 cases, 20% were Grade I (well differentiated), 70% were Grade II (moderately differentiated) and 10% were Grade III (poorly differentiated).The most common histological grade observed was moderately differentiated (70%) followed by well differentiated (20%) and poorly differentiated (10%).

Table 10: Pattern of p16 staining in OSCC cases.

Pattern of staining	Frequency	Percentage (%)
Nuclear+ Cytoplasm positive	24	48
Only Cytoplasm positive	21	42
Negative staining	5	10
Total	**50**	**100**

Figure 16. Pattern of p16 staining in OSCC cases.

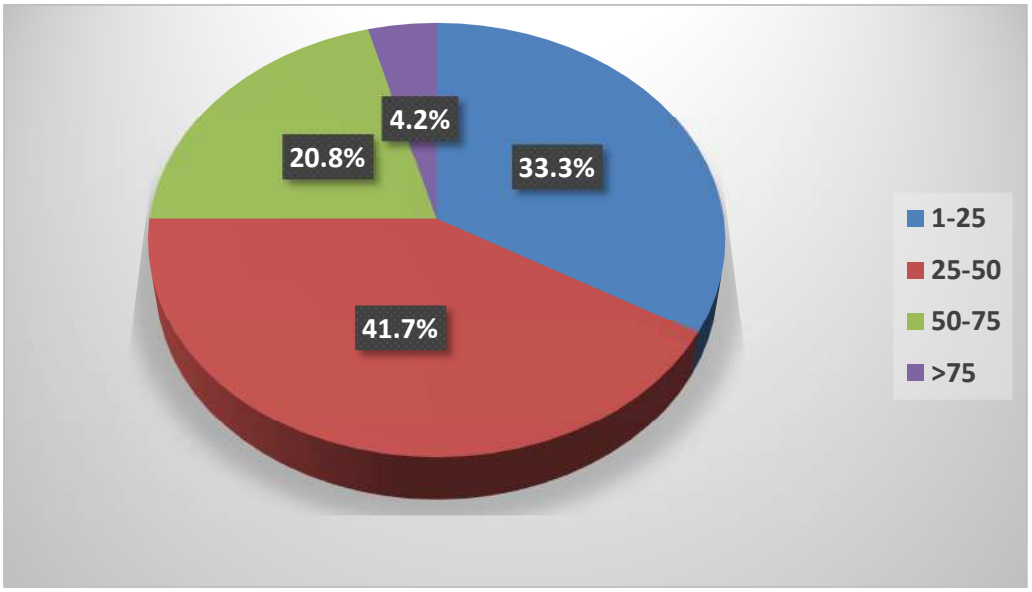


It was observed that out of 50 cases 48% cases were positive for p16 (nuclear and cytoplasm) inclusive of all histological grades while 42% cases showed only cytoplasm staining and 10% showed negative staining of p16.

Table 11: Percentage of positive tumour cells stained in p16-positive OSCC

Percentage (%) of tumor cells staining	Frequency	Percentage (%)
1-25	8	33.3
25-50	10	41.7
50-75	5	20.8
>75	1	4.2
Total	**24**	**100**

Figure 17. Percentage of positive tumour cells stained in p16-positive OSCC

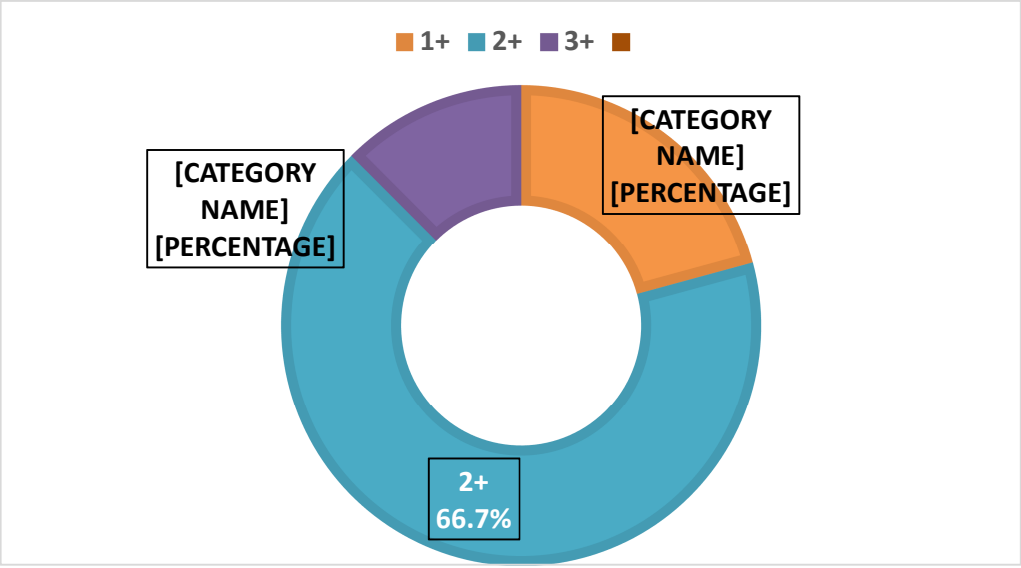


Among the p16 positive OSCC, it was observed that 42% showed 25–50% of tumour cells stained (Score 2), followed by 33% with 25% (Score 1), 21% of cases with 50–75% (Score 3) and only 4% showed >75% staining of tumour cells (Score 4).

Table 12: Tumor cell staining intensity grades in p16-positive OSCC

Intensity	Frequency	Percentage (%)
1+	5	20.8
2+	16	66.7
3+	3	12.5
Total	**24**	**100**

Figure 18. Tumour cell staining intensity grades in p16-positive OSCC

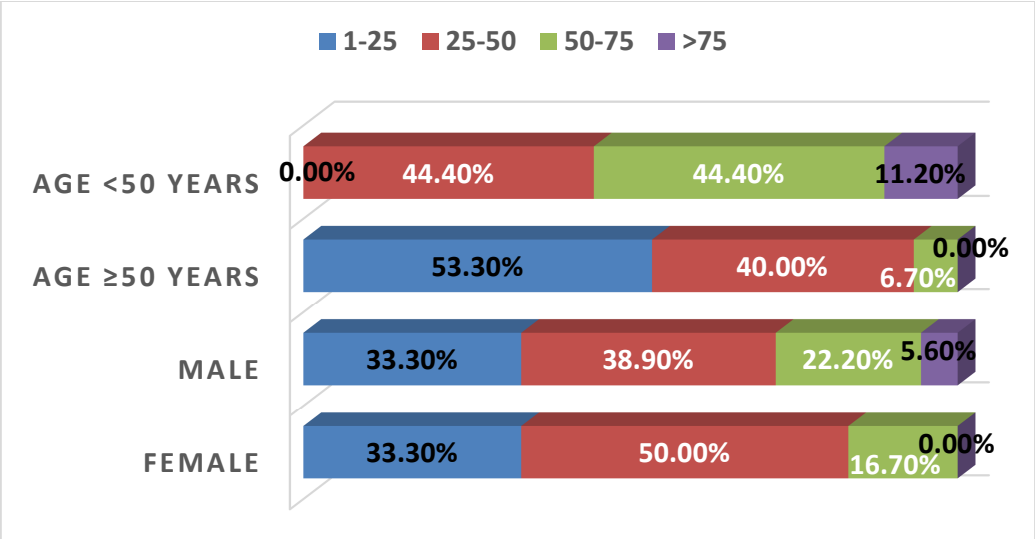


Out of p16 positive cases, 21% showed weak or bare or singly dispersed staining intensity (Grade1), 67% showed moderate or patchy intensity (Grade2), and 12% showed strong or diffuse intensity (Grade3).

Table 13: Association of patient's age and sex with percentage of positive (%) tumor cells among p16 positive OSCC.

Characteristics		**Percentage of positive Tumor Cells**				**p-value**
		1-25	**25-50**	**50-75**	**>75**	
Age	**<50**	0 (0.0%)	4 (44.4%)	4 (44.4%)	1 (11.2%)	
	≥50	8 (53.3%)	6 (40.0%)	1 (6.7%)	0 (0.0%)	**0.016**
Sex	**Male**	6 (33.3%)	7 (38.9%)	4 (22.2%)	1 (5.6%)	
	Female	2 (33.3%)	3 (50.0%)	1 (16.7%)	0 (0.0%)	0.912

Figure 19. Association of patient's age and sex with percentage of positive (%) tumor cells among p16 positive OSCC.



The association of patient's age with the percentage of positive tumour cells was statistically significant with a p value of 0.016, whereas the association of patient's sex with the percentage of positive tumour cells did not show statistical significance (p value-0.993).

Table 14: Association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry

Nuclear & cytoplasm staining of p16	Histological grading			p-value
	Poorly differentiated	Moderately differentiated	Well differentiated	
Absent	4 (15.4%)	16 (61.5%)	6 (23.1%)	0.304
Present	1 (4.2%)	19 (79.2%)	4 (16.7%)	
Total	5 (10.0%)	35 (70.0%)	10 (20.0%)	

Figure 20. Association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry

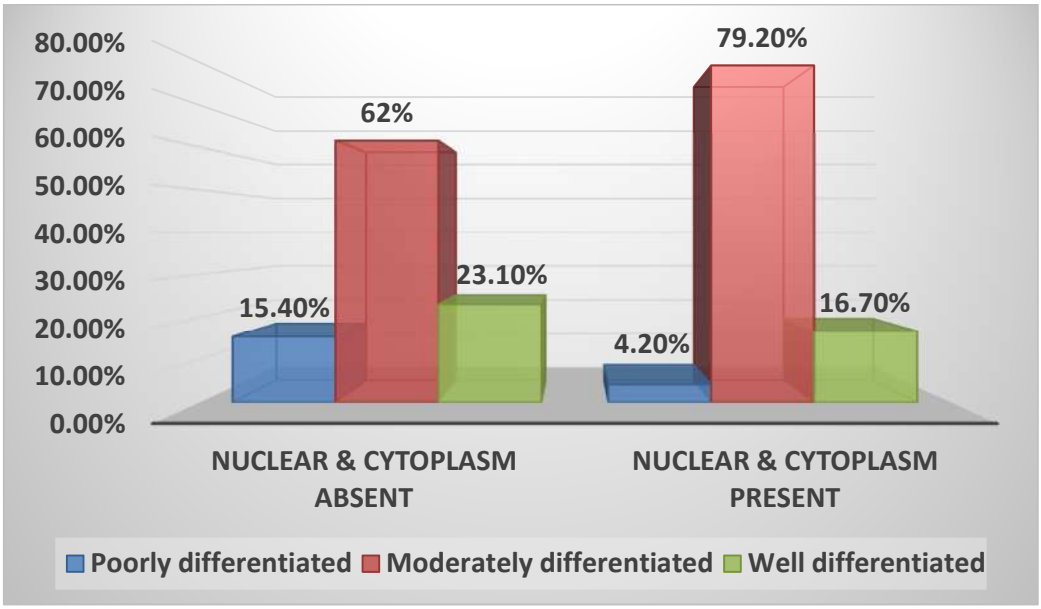
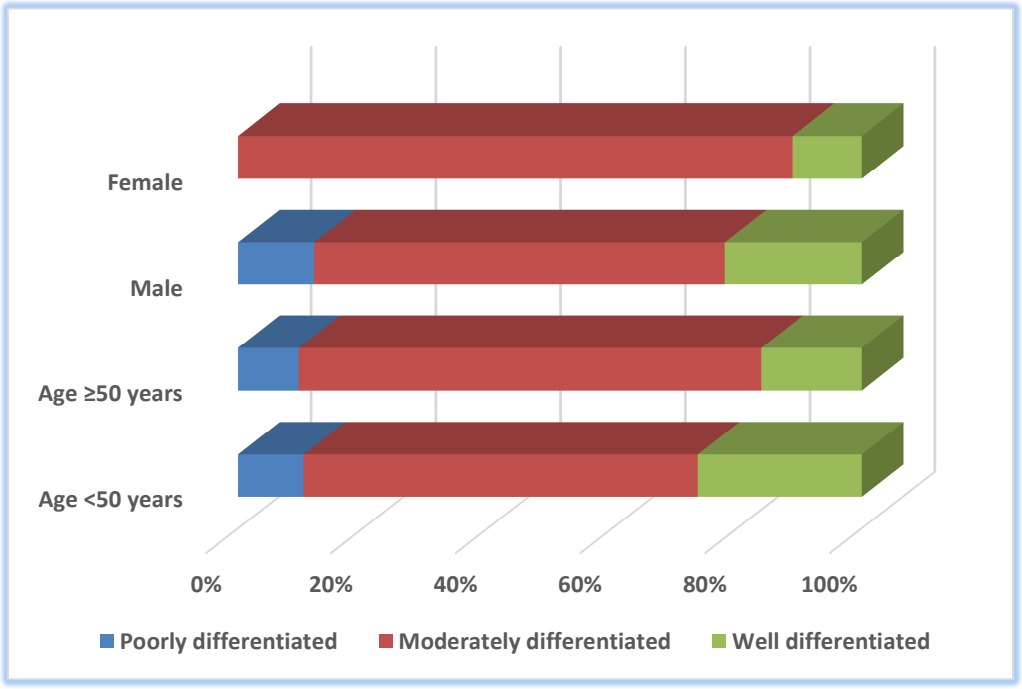


Table 9 illustrates the Association of human papilloma virus with the histological grading of oral carcinoma using p16 IHC. The association of human papilloma virus with the histological grading did not show statistical significance (p value- 0.304).

Table 15: Association of patient's age and sex with Histopathological grading of Oral carcinoma cases

Characteristics		H&E			p-value
		Poorly differentiated	Moderately differentiated	Well differentiated	
Age	**<50**	2 (10.5%)	12 (63.2%)	5 (26.3%)	
	≥50	3 (9.7%)	23 (74.2%)	5 (16.1%)	0.662
Sex	**Male**	5 (12.2%)	27 (65.9%)	9 (22.0%)	
	Female	0 (0.0%)	8 (88.9%)	1 (11.1%)	0.352

Figure 21. Association of patient's age and sex with histopathological grading of Oral carcinoma cases



The association between patient's age and sex with histopathological grading of Oral carcinoma did not show statistical significance.

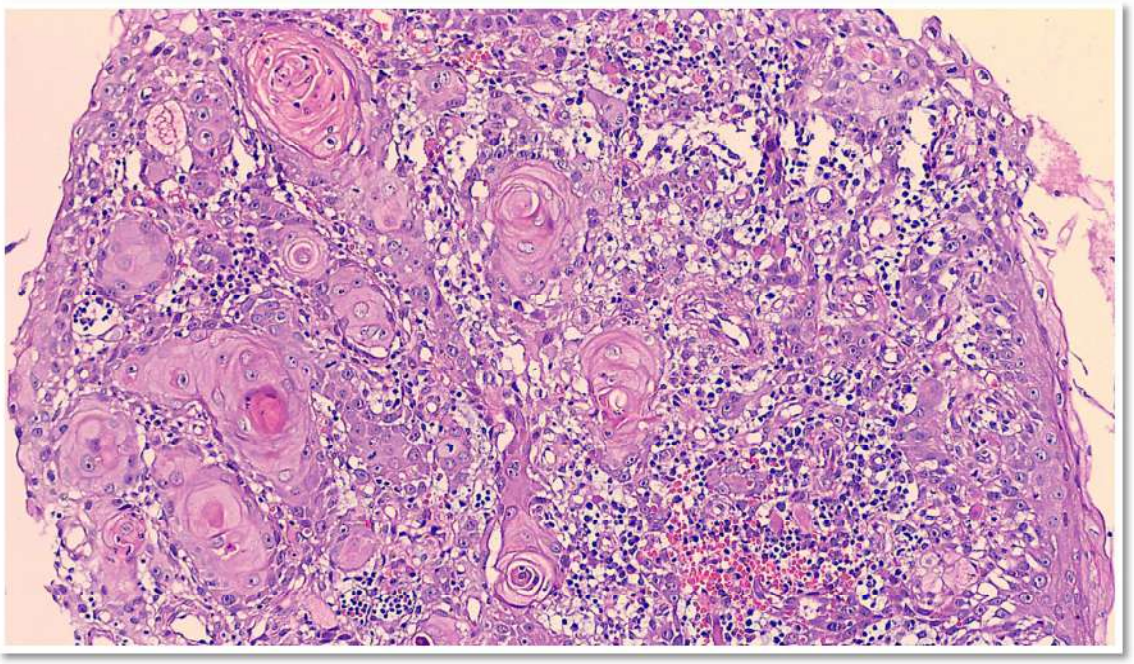


Figure 22. H &E, 100X- Well differentiated OSCC

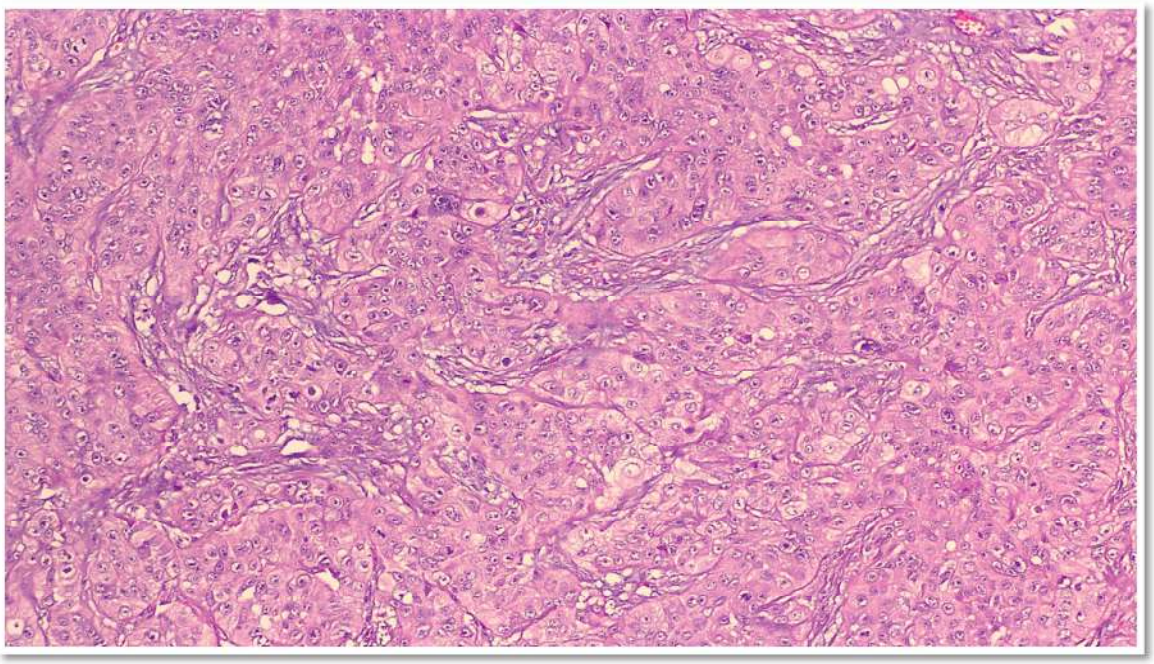


Figure 23. H &E, 200X- Moderately differentiated OSCC

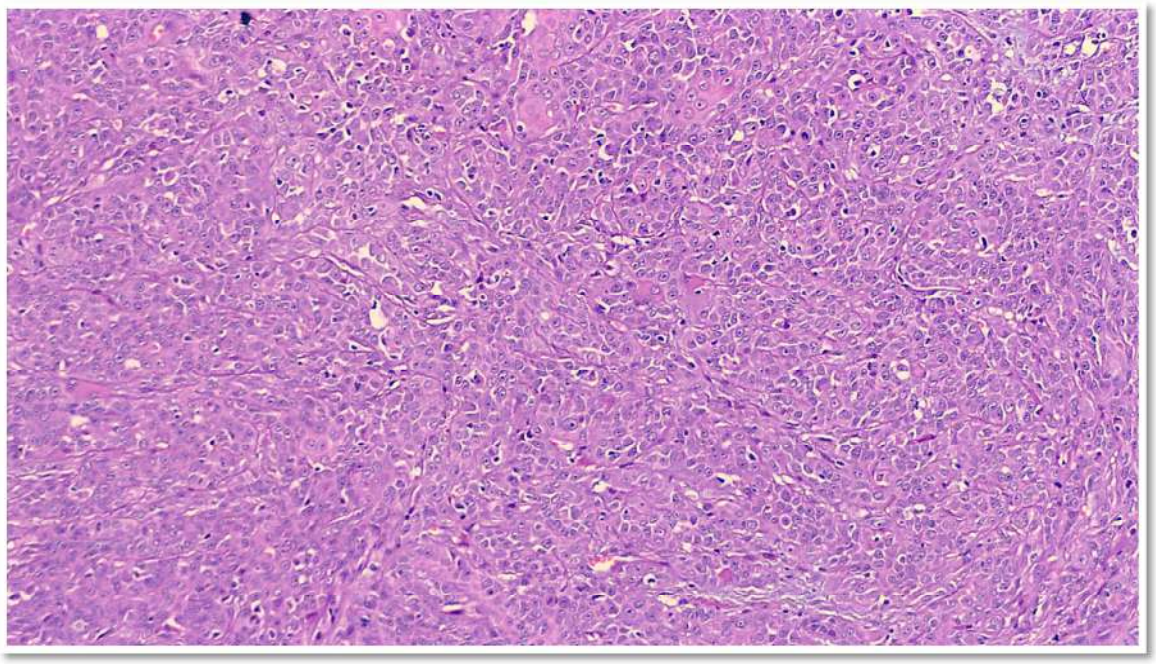


Figure 24. H &E, 100X- Poorly differentiated OSCC

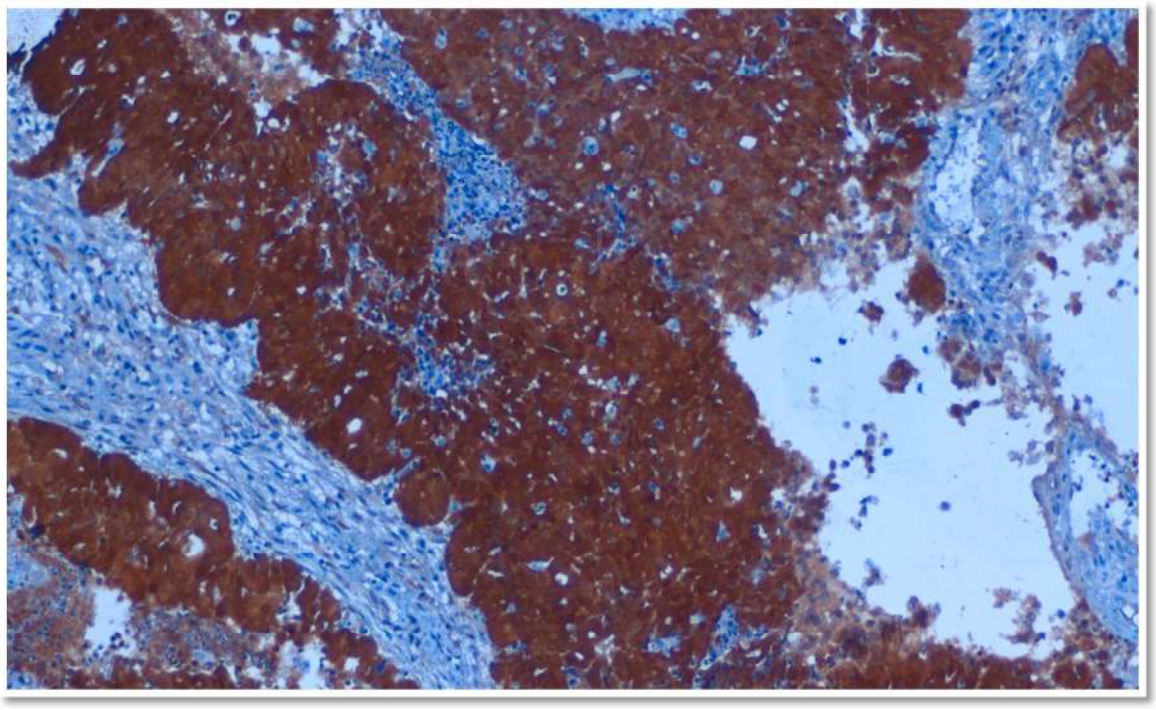


Figure 25. p16- Positive control (IHC, 100X)

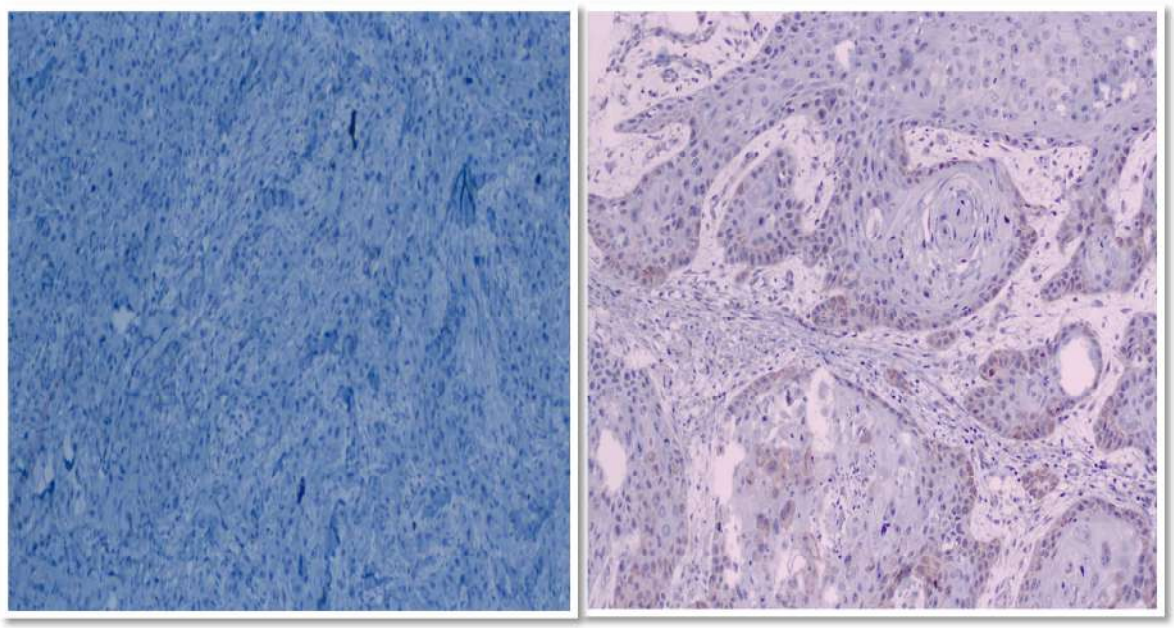


Figure 26. p16- Negative control (IHC, 100X)

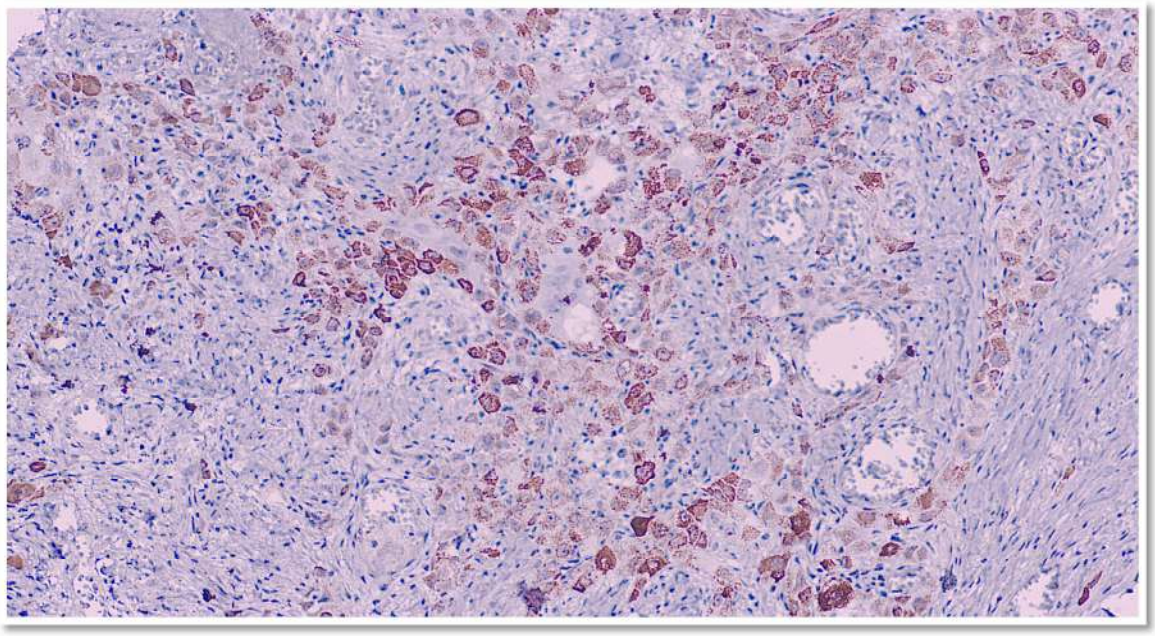


Figure 27. p16- Mild/Weak/Bare singly dispersed intensity (IHC, 100X)

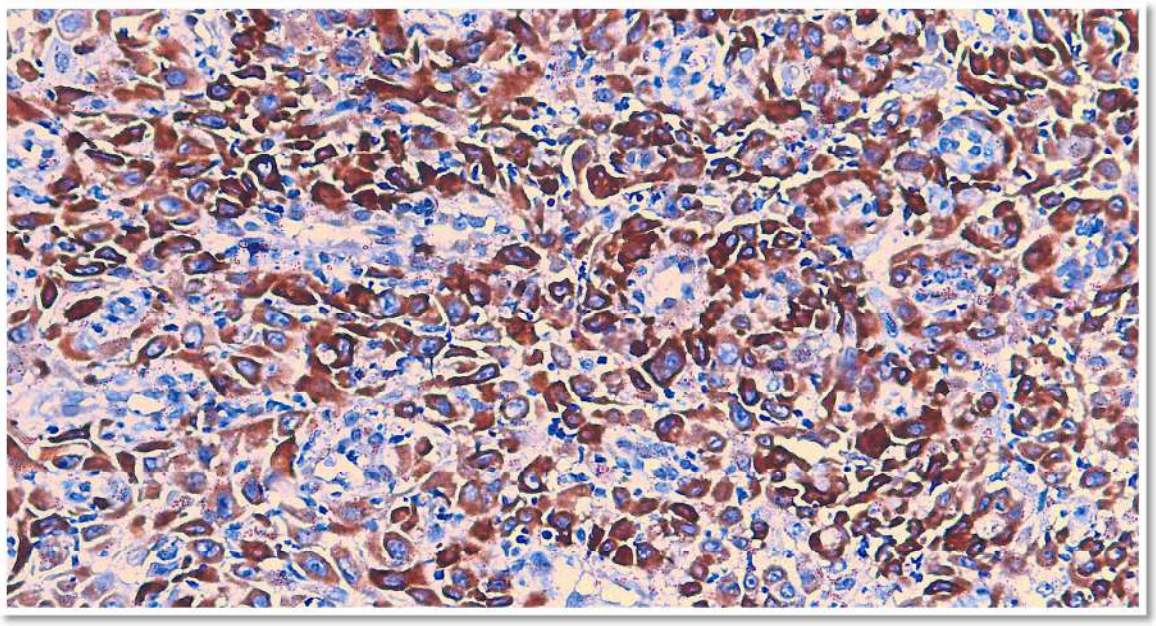


Figure 28. p16- Moderate/ Patchy intensity (IHC, 200X)

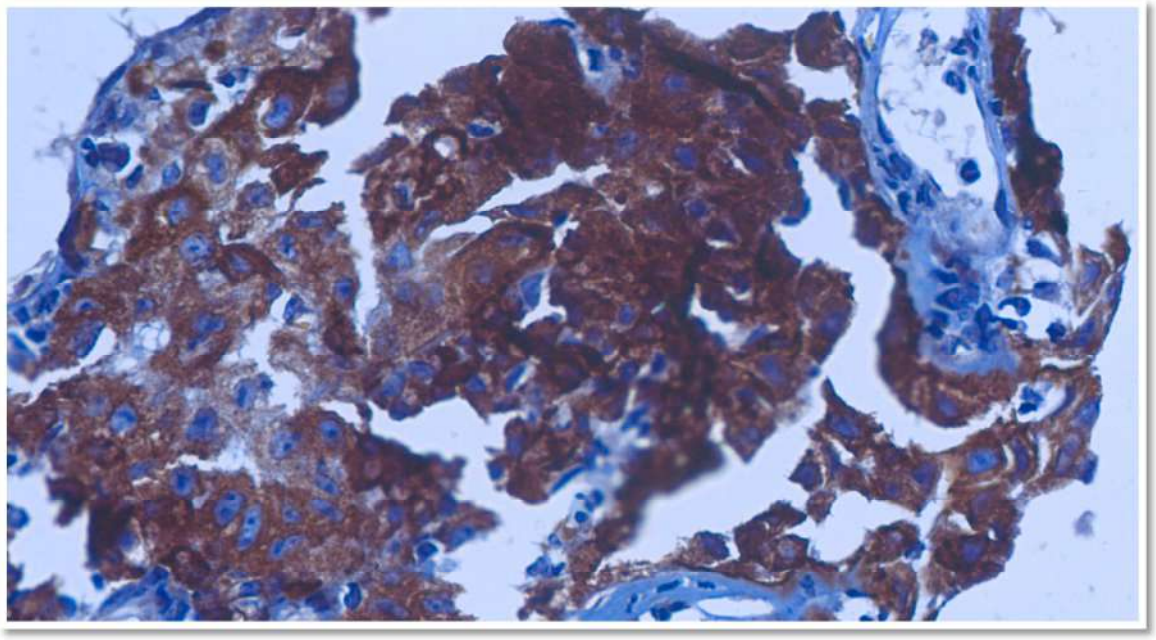


Figure 29. p16- Strong/Diffuse intensity (IHC, 400X)

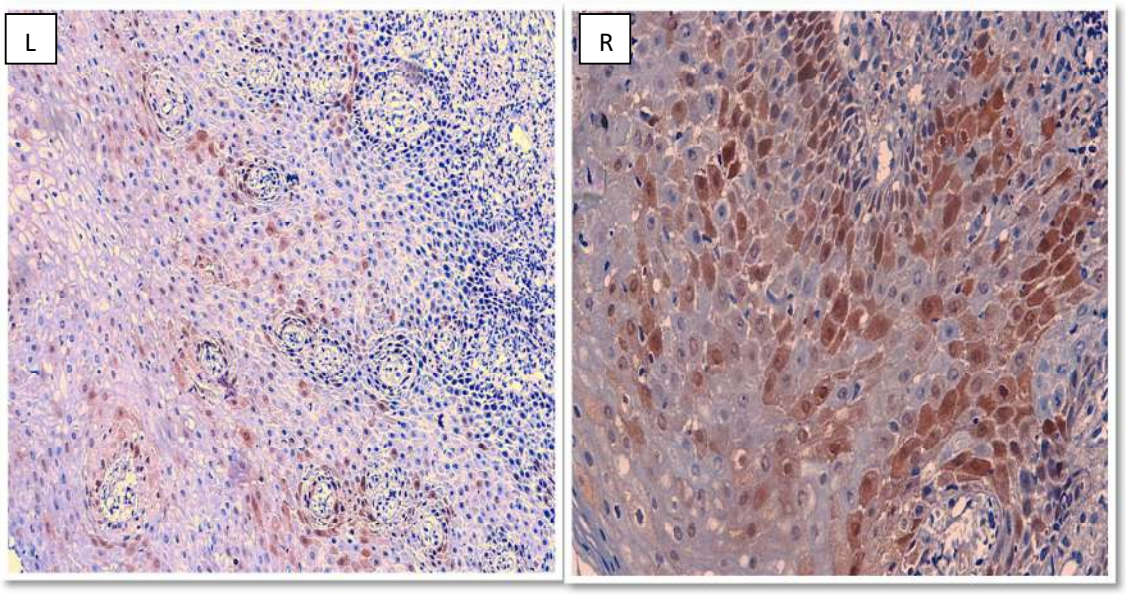


Figure 30. p16:1-25% of tumor cells staining

[IHC, Left (L)-50X, Right (R)- 100X]

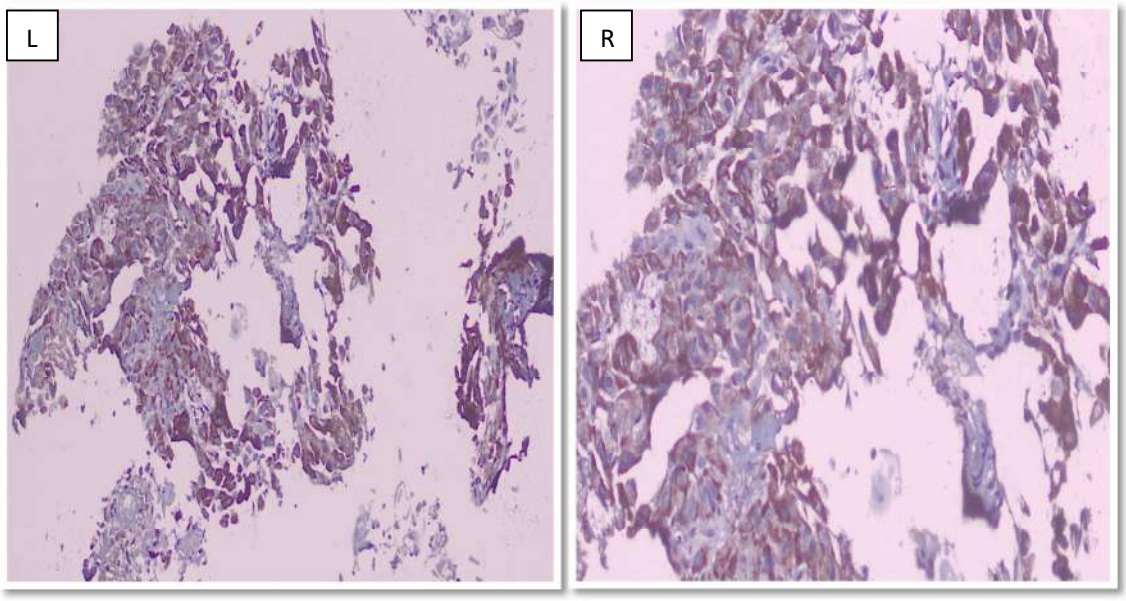


Figure 31. p16: 25%-50% of tumor cells staining

[IHC, Left (L)-50X, Right (R)- 100X]

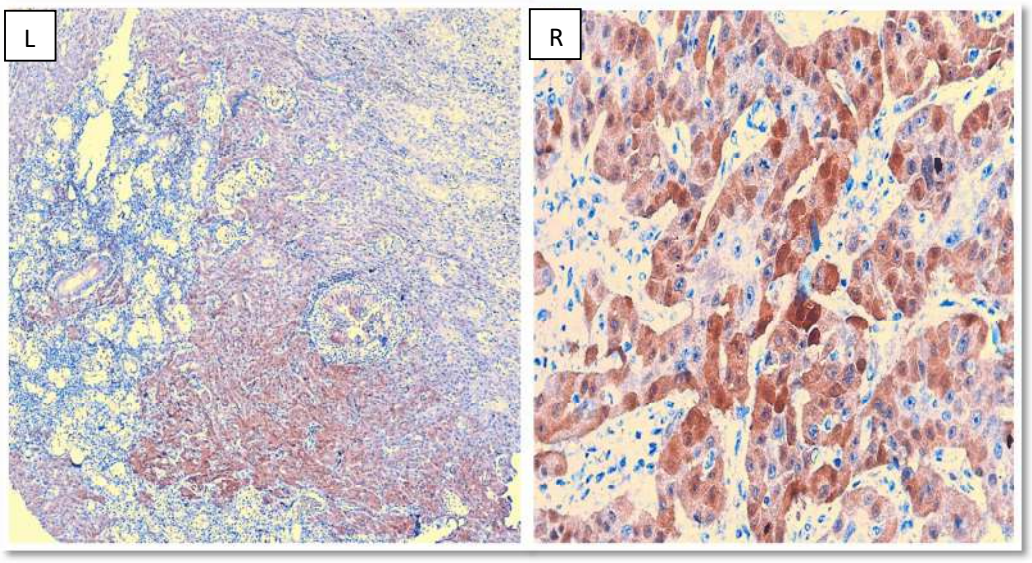


Figure 32. p16: 50%-75% of tumor cells staining

[IHC, Left (L)-50X, Right (R)- 200X]

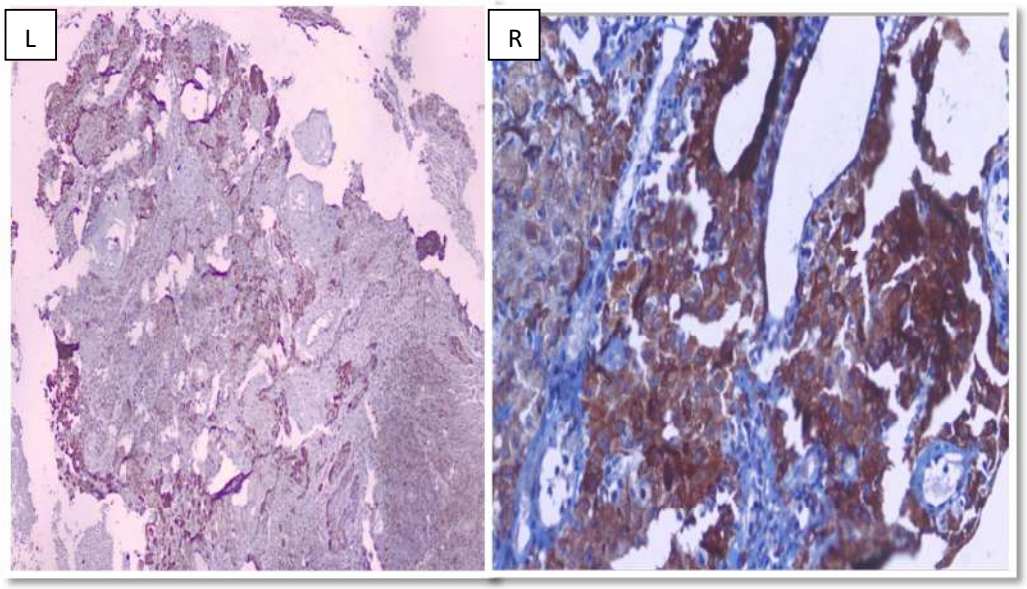


Figure 33. p16: >75% of tumor cells staining

[IHC, Left (L)-50X, Right (R)- 100X]

DISCUSSION

Oral cancer is a major worldwide health issue with high prevalence and fatality rate throughout the world[1-4,99]. Oral squamous cell carcinoma (OSCC) is considered as one of the most common type of malignancy in head and neck region[2]. According to WHO, current estimation shows 6,57,000 new cases of oral and pharyngeal cancer and deaths over more than 3,30,000 each year and South Central Asia being the highest-burden due to exposures to risk factors[100,101]. As per Global Cancer Observatory (GCO) data, estimated annual incidence of OSCC in 2020 was 3,77,713 cases worldwide, Asia (2,48,360) being the highest number recorded, followed by Europe(65,279) and North America(27,469). According to GCO, by 2040, the incidence and mortality of OSCC is predicted to increase by up to 40%[102].

OSCC accounts for 40-50% of all malignancies in India[2]. The primary known risk factor for oral cancer includes tobacco and alcohol consumption and work synergistically to increase the risk up to by 35%[103]. Recently Human papilloma virus (HPV) infection of high risk type has become evident and is linked to the development of oral cancers etiologically. HPV is a key risk factor to rule out while assessing a case of oral cancer and is highly associated with oral sex behaviour. HPV is an important risk factor in ruling out while assessing a case of oral cancers. These viruses are considered to be a carcinogenic infectious agent not only in cervical cancer but also in a proportion of oral cancers. The most commonly detected high-risk types are HPV16 and HPV18[2]

In oral cancers, HPV appears in the early stage of carcinogenesis or as an early initiator of proliferation. HPV associated cancers affect the cell cycle via the E6 and E7 viral oncoproteins that bind to and inactivate tumor suppressor proteins p53 and

pRb (retinoblastoma protein). It affects cell control of transcription, thereby promoting the malignant transformation of HPV infected cells leading to p16 overexpression, which is easily detected by immunohistochemistry (IHC)[1].

In 1985, the first reports of HPV types in HNSCC were published[104,105]. Since then, it has been shown that there is evidence that p16 overexpression can be used as a surrogate marker for HPV in HNSCC because HPV type 16 contributes to the loss of control over this suppressor protein[106,107]. HNSCC has been categorised as HPV positive and HPV negative disease and has been observed that HPV positive HNSCC has overall better survival rate than the HPV negative disease. Also, enhanced sensitivity to chemoradiation is observed with HPV positive OSCC[108-110].

Our study was done to detect human papilloma virus infection in cases of oral carcinoma using p16 immunohistochemistry and to determine the association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry. A total of 50 cases of OSCC was evaluated for the p16 overexpression.

Table 16: Comparing mean age for OSCC with other studies

Study	**Mean Age (years)**
Deng Z et al.	61.8
Caihua Liang et al.	56.4 ± 9.2
Pandey P et al.	46.5 ± 14.4
Ralli et al.	54.3± 11.3
Present study	**53.7± 11.7**

In the present study, age of the patients ranged from 31 to 70 years with a mean age of 54±12 years. 62% of cases were more than 50 years old and 38 % were below 50 years.

When compared to Deng Z et al. and Liang C et al. studies and Pandey P et al., the mean age for OSCC were 54±12, 61.6, 56±9 *and* 47 ±14 respectively[93,97,111,]. The mean age of the present study is similar to study done by Ralli et al.[112].

Table 17: Comparison of sex distribution of OSCC patients with other studies.

Liang C et al. study			
Sex of the patients	Intensity		p-value
	Negative	Positive	
Male	120 (72.3%)	46 (27.7%)	
Female	59 (88.1%)	8 (11.9%)	**0.01**
Total	179 (77%)	54 (23%)	233 (100%)
Kanyilmaz G et al. study			
Sex of the patients	Intensity		p-value
	Negative	Positive	
Male	69 (95%)	47 (82%)	
Female	4 (5%)	11 (18%)	**0.01**
Total	73 (56%)	58 (44%)	131 (100%)
Deng Z et al. study			
Sex of the patients	Intensity		p-value
	Negative	Positive	

Male	103 (81.1%)	24 (18.9%)	
Female	17 (73.9%)	6 (26.1%)	0.408
Total	120 (80%)	30 (20%)	150 (100%)
Ralli et al. study			
Sex of the patients	Intensity		p-value
	Negative	Positive	
Male	13 (20.3%)	51 (79.7%)	0.331
Female	3 (27.3%)	8 (72.7%)	
Total	16 (21.3%)	59 (78.7%)	75 (100%)
Present study			
Sex of the patients	Intensity		p- value
	Negative	Positive	
Male	23 (88.5%)	18 (75%)	
Female	3 (11.1%)	6 (25%)	0.902
Total	26 (52%)	24 (48%)	50 (100%)

In our study, male patients showed 75% of p16 overexpression as compared to female's 25% but there was no significant p value for the gender participants.

A study done by Liang C et al. and Kanyilmaz G et al. also found male patients more with p16 overexpression as compared to female with significant p value[111,113] This may be due to habit of consumption of tobacco either chewing or smoking and alcohol consumption which are more common among males. All these etiologies play a role in the development of OSCC.

Studies done by Deng Z et al., Ralli et al. and the present study showed p16 overexpression more in males without any statistical significant p value[93,112].

Table 18: Comparison of distribution of sites of OSCC patients with other studies.

Study	Site of Lesion
Bai XX et al.	Tongue>Gingiva> buccal mucosa
Prakash P et al.	Tongue >buccal mucosa > cheek
Pires FR et al.	Border of Tongue > Alveolar mucosa/Gingiva> Floor of mouth/ventral tongue
A. Sudhakaran et al.	Buccal mucosa> Lip> Alveolus
Present study	**Buccal mucosa> Tongue> Gingivobuccal sulcus**

In our study the most common site of involvement was buccal mucosa (50%) and the least was lip (4%) when compared to Bai XX et al., Prakash P et al. and Pires FR et al. studies most common site involved was tongue [89,114,115].

Another study done by A. Sudhakaran et al. also found buccal mucosa as the predominant site of involvement for OSCC which was concordance with my study[90]. This may be due to consumption of common forms of smokeless tobacco which include khaini, gutkha, betel quid with tobacco, and zarda.

World Health Organization (WHO) has also mentioned nearly 267 million adults (>15 years and above) in India are users of tobacco as per Global Adult Tobacco Survey India, 2016-17 and the most prevalent form used in India is the

above mention smokeless tobacco and smoking forms include cigarette, bidi and hookah[116].

Table 19: Comparing p16 overexpression in OSCC with other studies.

Study	Number of cases showing positivity	Percentage of cases showing positivity (%)
Tokuzen N et al.	10/100	10
de C. Ferreira C et al.	81/252	32
Hashmi A A et al.	64/144	44
Azizi SA et al.	26/28	93
Patil S et al.	26/30	87
Pandey P et al.	60/100	60
Sudhakaran A et al.	15/30	50
Present study	**24/50**	**48**

Studies done by Tokuzen N et al. and de C. Ferreira C et al. found very low prevalence of p16 positivity with 10% and 32% cases respectively[95,117].

whereas Azizi SA et al. and Patil S et al. studies showed a very high prevalence of p16 positivity with 93% and 87% respectively[2,96,].

Other studies done by Pandey P et al., Hashmi A et al., and Sudhakaran A et al. observed 60% 44% and 50% p16 positivity respectively which was concordance with the present study with 48% p16 positivity[90,91,97,].

This variability may be due to ethnicity, geography, exposure to tobacco products, alcohol consumption, greater oral sex exposure and number of samples analyzed in their study.

Another attributing factor causing this variability could be variations in the interpretation of the results due to different cut off values used while assessing or scoring the intensity, percentage of tumor cells stained as well as pattern of staining using p16 IHC and the types of antibodies used by different authors.

Table 20: Comparison of Association of HPV with the histopathological grading of oral carcinoma cases using p16 IHC with other studies

p16	Histological grading			**p-value**
Deng Z et al. study	Well differentiated	Moderately differentiated	Poorly differentiated	
Absent	11 (57.9%)	51 (81%)	58 (85.3%)	
Present	8 (42.1%)	12 (19%)	10 (14.7%)	**0.030**
Total	19 (12.7%)	63 (42%)	68 (45.3%)	
Meng et al. study	Histological grading			
	Well differentiated	Moderately differentiated	Poorly differentiated	**<0.05**
Absent	443 (31.9%)	798 (57.4%)	148 (10.7%)	
Present	24 (29.6%)	20 (24.7%)	37 (45.7%)	
Total	467 (31.8%)	818 (55.6%)	185 (12.6%)	
Ralli et al. study	Histological grading			
	Well differentiated	Moderately differentiated	Poorly differentiated	**0.045**

Absent	2 (3.5%)	51 (91%)	3 (5.5%)	
Present	4 (21%)	11 (58%)	4 (21%)	
Total	6 (8%)	62 (82.7%)	7 (9.3%)	
Pandey P et al. study	Histological grading			0.36
	Well differentiated	Moderately differentiated	Poorly differentiated	
Absent	28 (70%)	11 (27.5%)	1 (2.5%)	
Present	33 (55%)	24 (40%)	3 (5%)	
Total	61 (61%)	35 (35%)	4 (4%)	
Present study	**Histological grading**			
	Well differentiated	**Moderately differentiated**	**Poorly differentiated**	0.304
Absent	6 (23.1%)	16 (61.5%)	4 (15.4%)	
Present	4 (16.7%)	19 (79.2%)	1 (4.2%)	
Total	**10 (20.0%)**	**35 (70.0%)**	**5 (10.0%)**	

A study done by Meng et al. found p16 positive cases more in poorly differentiated OSCC (Grade3) whereas Pandey P et al. found maximum cases positive in well differentiated OSCC (Grade1)[93,98].

In the present study the most common histological grade observed was moderately differentiated (70%) followed by well differentiated (20%) and poorly differentiated (10%) and the maximum number of p16 positive cases was found in histological Grade 2 which was similar to the studies done by Deng Z et al. and Ralli et al.[93,112].

Studies done by Deng Z et al., Meng et al. and Ralli et al. showed a significant association of human papilloma virus with the histological grading of OSCC using p16. ($p< 0.05$)[93,98,112]

However, in our study there was no statistically significant association of HPV with the histopathological grading of oral carcinoma cases using p16 IHC (p value-0.304) which is in concordance with study done by Pandey P et al. and Yuen et al.[97,118].

These dissimilarity findings could be attributed to the difference in sample size, duration of study, geographic distribution of tumor, different types of antibodies used while staining and scoring criteria used by different authors.

CONCLUSION

The present study revealed p16 overexpression in 24/50 (48%) cases of OSCC with maximum cases showing 25-50% of tumor cells having a moderate degree of staining intensity. This finding suggests a low prevalence of HPV infected OSCC in this region. Our study also showed an association of patient's age with percentage of positive tumor cells among p16 positive OSCC (p value- **0.016**).

p16 IHC is an important biomarker, particularly for high-risk HPV types infection, which makes it useful in evaluating HPV associated squamous carcinoma. Therefore, knowing the HPV status by IHC is a more feasible, easy, cost effective, and reproducible test as compared to HPV polymerase chain reaction (PCR) and could impact patient management and survival and the development of prophylactic vaccines based on their viral capsids.

However, our study also concluded that there is no statistically significant association between the expression of p16 and the histopathological grading of OSCC.

LIMITATIONS-

- Sample size was limited in our study (50). Our study did not reveal a significant association between p16 and the histopathological grading of OSCC. The study on higher numbers would probably be able to find an association if any.
- Our study considered p16 positive equivalent to HPV DNA and did not compare it with PCR, which is the gold standard test for HPV DNA due to the higher cost of PCR. A Comparative study with PCR would be confirmatory.

SUMMARY

- This cross-sectional study was done at Histopathology laboratory at KLE'S DR. PRABHAKAR KORE CHARITABLE HOSPITAL and KLE'S DR. PRABHAKAR KORE HOSPITAL and MEDICAL RESEARCH CENTER, BELAGAVI by collecting data and blocks of Oral squamous cell carcinoma cases from January 2021 and December 2021.
- The objectives of the study were to detect human papilloma virus infection in cases of oral carcinoma using p16 immunohistochemistry and to determine the association of human papilloma virus with the histological grading of oral carcinoma using p16 immunohistochemistry.
- The study took into account 50 samples of oral squamous cell cancer.
- Of the 50 OSCC cases, there were 41 male patients and 9 female patients. The patients' ages ranged from 31 to 71 years old, with a mean age of 54 ± 12 years.
- p16 overexpression was found in 24/50 (48%) of the OSCC patients in the current study. With a sex ratio of 4.5:1, p16 positive male OSCC cases were more common than female p16 positive OSCC cases.
- The most frequent site of occurrence for p16 positive cases of OSCC was buccal mucosa, followed by tongue and alveolus.
- It was discovered that the majority of cases showed moderate degree of staining intensity (Score 2) with 25-50% of tumour cells staining, followed by 25%, 50-75%, and only one case showed >75% staining of tumour cells. This was determined by studying the immunohistochemical expression of

p16 when nuclear and cytoplasmic staining were taken into account. Only cytoplasmic staining and total absence were considered negative.

- The association of patient's age with percentage of positive tumor cells among p16 positive OSCC was found statistically significant (p value- **0.016**)
- Out of 50 cases of OSCC, the histological grade with the highest frequency was moderately differentiated (70%), followed by well differentiated (20%) and poorly differentiated (10%) cases.
- No statistically significant association was found between human papilloma virus infections with the histological grading of OSCC using p16 IHC. (p value- 0.304)
- Similarly, no association was found between a patient's age and sex with histopathological grading of p16 positive oral carcinoma cases.

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ANNEXURE - I- INFORMED CONSENT FORM

"DETECTION OF HUMAN PAILLOMA VIRUS INFECTION IN CASES OF ORAL CARCINOMA USING p16 IMMUNOHISTOCHEMISTRY"

Principal Investigator: Dr.

Guide: Dr.

Purpose of the study: The purpose of this study is to detect the HPV infection in cases of oral carcinoma using p16 immunoexpression and its association with histological grading .You are being asked to enroll in this study as you are eligible for participation in this study. If you are diagnosed with oral carcinoma, you will be included in this study.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge. If you agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to detect HPV infection in cases of oral carcinoma using p16 immunoexpression. Knowing the HPV status by IHC is a more feasible, easy, cost effective, reproducible test than HPV polymerase chain reaction (PCR) and could impact patient management and survival and the development of prophylactic vaccines based on their viral capsids.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you can later change your mind and withdraw from the study. The study doctor may terminate your participation in this study anytime.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorization to publish results: The results of this study would be forwarded to the KAHER, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

Questions: In case you have any questions related to the study in future you can contact:

1. If you have any queries about your rights as a study subject, you may call Dr. Harsha Hegde, Chairman of J.N. Medical College, Institutional Ethical Committee & scientist D, ICMR, National Institute of Traditional Medicine, Phone No- 9480422500, at J.N. Medical College, Belagavi.

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read or it has been read to me this entire consent form and have had all my questions answered.

Name of the participant: (Signature/thumbprint)

Name of the witness: (Signature/thumbprint)

Name of the investigator: (Signature)

Date:

ANNEXURES II – PROFORMA

PROFORMA FOR ORAL CARCINOMA

PATIENT HISTORY

Name: Age: Sex: IP no.:

History:

Clinical diagnosis with stage:

Type of sample: Biopsy/Complete excision/Any other procedure

Gross:

Size:

Extension:

Lymph node involvement:

No. of Lymph nodes involved:

Groups of lymph node:

Microscopy:

1. Histopathological diagnosis and grade:
2. IHC staining:
    - Pattern of staining:
    - Percentage of tumour cells stained by p16:
    - Score based on staining intensity:

ANNEXURE- III

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

1. Deparaffinize in Xylene I and II and III changes. (III change use warmed xylene) (5 minutes in each)
2. Rehydrate using
    a. Absolute ethanol 100% (5 minutes)
    b. Absolute Ethanol 100% (5 minutes)
3. Rinse in distilled water (5 minutes)
4. Rinse in running tap water (5 minutes)
5. Stain in Harris's haematoxylin by progressive method (2 minutes) Fresh and filtered
6. Rinse in running tap water (20 minutes)
7. Decolorize in 1% acid alcohol (1 second)
8. Rinse well in tap water (5 minutes)
9. Immerse in hot water bath, 55˚C for blueing (3 seconds)
10. Rinse in tap water (5 minutes)
11. Counterstain in Eosin (15 seconds)
12. Dehydrate with absolute alcohol 100% (2-4 dips)
13. Clear in xylene I and II (5 minutes)
14. Mount with DPX.

Stock solution – Eosin:

Stock – 1% aqueous Eosin – Y

Stock – 1% aqueous Phloxin B

Working Solution – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial acetic acid

Working Solution – Hematoxylin

Harris Hematoxylin, 1 litre

Working solution – 0.25% Acid alcohol

95% Ethanol, 2578 ml

dH2O, 950 ml

HCl, 9ml

Result: Nuclei – blue, cytoplasm – pink, RBCs – red.

Reference: Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. &th Ed., China, Churchill Livingstone; 2013: p173-187.

ANNEXURE- IV

IMMUNOHISTOCHEMICAL STAINING PROTOCOL FOR p16

1. Cut the sections at approximately 3-4 µm thickness in poly L Lysine coated slides.
2. Bake the sections at 37 degree celsius overnight. Before test bake it at 60 degree celsius for 1 hour
3. Deparaffinise steps-
   i. Xylene I- 10 minutes
   ii. Xylene II- 10 minutes
   iii. Absolute alcohol I- 10 minutes
   iv. Absolute alcohol II- 10 minutes
   v. Rinse in water- 5 minutes
   vi. Rinse in distilled water- 1 minute
4. Antigen retrieval by (TRIS buffer +EDTA)- Buffer solution
   (Required amount of buffer is prepared and cook the slides in pressure cooker for 3 whistles)
5. Cooling of sections to room temperature for 15 minutes.
6. Wash with wash buffer 2 times with gap of 30 seconds each
7. Treatment with 3% hydrogen peroxide for 8-10 minutes to block endogenous peroxidase.
8. Wash with water buffer 3 times with a gap of 30 seconds
9. Treatment with primary monoclonal antibody for p16 protein (biogenex) for 45 to 60 minutes in closed chamber at room temperature
10. Wash with wash buffer 3 times with gap of 30 seconds each

11. Treatment with polymer Horseradish peroxidase (HRP) for 25 to 30 minutes in closed chamber at room temperature.
12. Wash with wash buffer 3 times with gap of 30 seconds each
13. Treatment with Diaminobenzidine (DAB) substrate (secondary antibody) for 10 minutes to give brown colour to antigens.
14. Wash with water for 2 minutes
15. Wash with distilled water for 1 minute.
16. Counter stain with Harris haematoxylin for 3 minutes.
17. Blueing in warm water- 1minute
18. Clearing with xylene for two minutes. Dry the slides and mount with DPX

PREPARATION OF REAGENTS

1. Antigen retrieval Buffer

TRIS EDTA Buffer- pH: 8.5 to 9.0

Preparation:

TRIS Base- 1.21 gram

EDTA (atomic number: 372)- 0.37 gram

Dissolve in 1000ml of water

2. Wash buffer

TRIS BUFFERED SALINE (TBS)- pH: 7.2 to 7.6

Preparation:

TRIS Base- 8.6 gram

NaCl- 9.6 gram

Dissolve in 1000ml of water.

Adjust pH by using concentrated HCl

ANNEXURE- V

KEY TO MASTER CHART

M	-	Male
F	-	Female
GBS	-	Gingivobuccal mucosa
MT	-	Molar tooth
BM	-	Buccal Mucosa
(R)	-	Right
(L)	-	Left
WLE	-	Wide local excision
PMMC	-	Pectoralis major myocutaneous flap
MRND	-	Modified radical neck dissection
SOHND	-	Supraomohyoid neck dissection
RND	-	Radical neck dissection
ND	-	Neck dissection
H&E	-	Hematoxylin and Eosin
PD	-	Poorly differentiated
MD	-	Moderately differentiated
WD	-	Well differentiated
%	-	Percentage
+ve	-	Positive
TC	-	Tumour cells
N	-	Nuclear
C	-	Cytoplasm

ANNEXURE- VI - MASTER CHART

Sl. No.	Sample No.	Age	Sex	Tumor site	Surgery performed	H&E Diagnosis	IHC expression of p16		
							N & C staining	% of p16 +ve TCs	Intensity
1	2/21	49	M	GBS + MT + BM	Punch biopsy	MD	N + C	51-75	2+
2	64/21	59	M	Alveolus + GBS	Composite resection + (L) hemi-mandibulectomy + composite MRND & SOND	MD	Nil	Nil	Nil
3	237/21	60	M	Alveolus	Punch biopsy	MD	N + C	<25	2+
4	240/21	60	M	BM + Tongue	Right WLE of BM & tongue & (R) hemimandibulectomy + RND	MD	C	<25	1+
5	435/21	52	M	Tongue	Incisional biopsy	MD	C	<25	1+
6	576/21	60	M	GBS	Composite resection + PMMC flap reconstruction	PD	N + C	25-50	2+
7	620/21	55	M	BM	Incisional biopsy	WD	C	<25	1+
8	653/21	63	M	Alveolus + GBS + BM	(R) commando procedure with PMMC flap	MD	N + C	25-50	2+
9	698/21	60	M	Alveolus+ BM	Punch biopsy	WD	N + C	25-50	2+
10	723/21	39	M	BM	Punch biopsy	MD	N + C	>75	3+

11	743/21	46	M	BM	WLE with PMMC flap	WD	C	<25	1+
12	744/21	78	F	Lip	Punch biopsy	MD	N + C	25-50	2+
13	791/21	44	M	Tongue	(R) Hemiglossectomy +(R) MRND	WD	C	<25	1+
14	819/21	53	M	Alveolus + GBS + BM	Composite resection + (L) hemimandibulectomy + (L) MRND + PMMC flap	MD	C	25-50	1+
15	959/21	61	M	Tongue	WLE + (L) hemimandibulectomy + PMMC flap reconstruction	WD	Nil	Nil	Nil
16	1069/21	56	M	BM + MT + GBS	Hemimandibulectomy + WLE of BM + PMMC flap reconstruction	PD	C	<25	1+
17	1083/21	70	F	BM + GBS	Composite resection of (L) GBS + MRND + PMMC flap reconstruction	WD	C	<25	1+
18	1105/21	64	F	BM	Punch biopsy	MD	N + C	25-50	2+
19	1473/21	45	M	Tongue	Composite resection of (L) tongue + segmental mandibulectomy	PD	C	25-50	1+
20	1587/21	70	M	ongue	WLE + ND	WD	N + C	<25	2+
21	1600/21	48	M	Lip	WLE + ND + Reconstruction	MD	C	51-75	1+
22	1652/21	38	M	BM	Bite resection + Hemimandibulectomy + Segmental maxilllectomy	WD	C	<25	1+

23	1721/21	34	M	BM	Commando operation + Reconstruction + ND	MD	N + C	51-75	2+
24	1972/21	48	M	BM	Composite + PMMC flap reconstruction	MD	N + C	51-75	2+
25	2031/21	70	F	Tongue	WLE + SOHND	MD	N + C	<25	1+
26	1746/21	44	M	Tongue	WLE + ND	WD	Nil	Nil	Nil
27	2231/21	70	M	Tongue	Punch biopsy	WD	N + C	<25	1+
28	2307/21	52	M	BM	WLE + Marginal mandibulectomy + Reconstruction + (L) ND	MD	N + C	25-50	1+
29	2353/21	62	M	Tongue	WLE + SOND	MD	C	<25	1+
30	2490/21	58	M	Tongue	WLE + MRND	MD	C	<25	1+
31	2549/21	66	M	BM	WLE	MD	C	25-50	1+
32	2373/21	69	M	BM	Bite resection + RND + PMCC flap	MD	C	<25	1+
33	2704/21	48	F	BM	WLE + Hemimandibulectomy + MND	MD	C	<25	1+
34	2722/21	33	M	BM	Punch biopsy	MD	N + C	51-75	3+
35	2738/21	65	F	Tongue	WLE + extended SOHND + Glossectomy	MD	Nil	Nil	Nil
36	2739/21	44	M	BM	Composite resection	MD	C	<25	2+

37	2752/21	70	F	Tongue	Punch biopsy	MD	N + C	<25	3+
38	2804/21	42	M	BM	WLE (L) BM+ L SOHND	MD	N + C	25-50	2+
39	2893/21	58	M	Tongue	Punch biopsy	MD	N + C	<25	2+
40	2886/21	59	M	BM	Punch biopsy	MD	C	<25	1+
41	2933/21	43	M	BM	WLE	MD	C	25-50	1+
42	2963/21	60	M	BM	(L) BM WLE + Hemimandibulectomy + MRND	MD	Nil	Nil	Nil
43	2998/21	60	M	Lip	WLE of upper lip + left angle of lip	MD	N + C	25-50	1+
44	3000/21	56	M	BM	Punch biopsy	MD	N + C	<25	2+
45	3059/21	26	M	Tongue	WLE (R) Tongue + Hemimandibulectomy + PMMC flap	MD	N + C	25-50	2+
46	3098/21	56	F	Tongue	Punch biopsy	MD	N + C	51-75	2+
47	3130/21	53	M	BM	(L) Commando + PMMC flap + MND	MD	C	<25	1+
48	3212/21	40	M	GBS	(L) BM WLE + Hemimandibulectomy + Alveolectomy +MRND	PD	C	<25	1+
49	3237/21	31	M	Tongue	WLE + (R) MRND + (L) SND	WD	N + C	25-50	2+
50	3643/21	42	F	Tongue	Punch biopsy	MD	N + C	25-50	1+