A Study of Distribution of p53, Cyclin D1 and CD44 in Oral Squamous Cell Carcinoma and its Correlation with Grading and NodAL Metastasis

Dissertation Submitted in

partial fulfillment of the regulations required for the award of

M.D. DEGREE IN PATHOLOGY –BRANCH III

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DECLARATION

I hereby declare that the dissertation entitled **"A Study of Distribution of p53, Cyclin D1 and CD44 in Oral Squamous Cell Carcinoma and its Correlation with Grading and Nodal Metastases"** was done by me in the Department of Pathology, Coimbatore Medical College under the guidance and supervision of **Dr. M. Murthy, M.D.,** Additional Professor, Department of Pathology, Coimbatore Medical College.

This dissertation is submitted to The Tamilnadu Dr.MGR Medical University, Chennai towards the partial fulfillment of the requirement for the award of M.D.Degree in Pathology.

Place: **Dr. K. Rohini**

Date:

CERTIFICATE

This is to certify that the dissertation entitled **"A Study of Distribution of p53, Cyclin D1 and CD44 in Oral Squamous Cell Carcinoma and its Correlation with Grading and Nodal Metastases"** is a record of bonafide work done by **Dr. K. Rohini** in the Department of Pathology, Coimbatore Medical College, Coimbatore and submitted in partial fulfillment of the requirements for the award of M.D. Degree in Pathology by The Tamilnadu Dr.MGR Medical University, Chennai. This work has not previously formed the basis for the award of a degree or diploma.

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ETHICS COMMITTEE

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Period of Study : 2007 - 2010

College : Coimbatore Medical College

Dissertation Topic : A Study of distribution of p53, cyclin D1 and CD 44 in oral squamous cell carcinoma and its correlation with grading and nodal metastasis.

The Ethics Committee, Coimbatore Medical College has decided to inform that your Dissertation is accepted / ~~Not accepted~~ and you are permitted / ~~Not Permitted~~ to proceed with the above Study.

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Date : 05.03.08

Secretary
Ethics Committee

INTRODUCTION

Squamous cell carcinoma (SCC) of the mouth constitutes the sixth most common cancer worldwide but the third most common in developing countries.[1] There is evidence of an increase in incidence rate and mortality as a result of this disease in recent years, particularly in young adults in Central Europe.[2] Despite advances in treatment modalities, the prognosis of this cancer is still very poor and has not changed over the past few decades.

Biological phenotypes of cancer greatly affect the clinical outcomes of patients with the disease. If such biological characteristics of cancer could be predicted before treatment, it would be possible to select more effective and suitable treatment for each cancer. Recent studies have clarified that a variety of molecular events play extremely important roles in not only tumor development but also tumor progression. Consequently, special attention has turned to molecular markers as a possible means for obtaining useful information to predict aggressive phenotypes of tumors.[3]

Since the current TNM staging system is also inadequate to accurately classify the patients of oral SCC in terms of prognosis, it is important to look for new biological prognostic markers that might add information about the aggressiveness of the tumors and treatment response. So, the interest lies in studying the molecular markers involved in cell cycle regulation of tumor cells p53 and cyclin D1. The p53 tumor-suppressor gene regulates cell cycle progression through induction of apoptosis at the G1/S checkpoint.[4,5]

Immunohistochemical p53 protein expression is based on the prolonged half-life of the mutant protein compared to the wild-type.[6] Cyclin D1 plays a central role in the G1/S cell cycle transition and responses to cytotoxic stimuli.[7, 8] We were also interested in the expression of CD44 molecule that has been correlated to carcinogenesis and aggressive biological behavior of several malignant tumors. CD44 is a polymorphic family of cell surface proteoglycans and glycoproteins implicated in cell-cell and cell-matrix adhesion interactions, lymphocyte activation and homing, cell migration and tumor metastasis.

In view of the prospective impact of multiple molecular marker accumulation on tumor progression, multiple-marker testing could provide us with more useful information for our definition of the biological behavior than single marker expression. So, in our study we have analyzed the expression of cell cycle regulatory proteins p53 and cyclin D1, cell adhesion molecule CD44 using immunohistochemistry in oral squamous cell carcinoma and its variant verrucous carcinoma. We also studied the impact of expression of these markers on clinico-pathological features like histological grading of the tumor and cervical node metastasis.

AIM OF THE STUDY

- To study the abnormal expression of cell cycle regulatory proteins particularly p53 and Cyclin D1 in oral squamous cell carcinoma using immunohistochemistry.
- To study the expression of cell adhesion molecule CD44 in oral squamous cell carcinoma using immunohistochemistry.
- To compare the expression of these markers in different histological grades of oral squamous cell carcinoma.
- To study the expression of these markers in verrucous carcinoma.
- To study the role played by the three molecular markers p53, cyclin D1 & CD44 in tumor grade and cervical node metastases.

NEED FOR THE STUDY

Oral SCC, an aggressive epithelial malignancy is posing a major threat to public health worldwide. In global terms oral cancer is the sixth most common malignancy associated with great morbidity and mortality.

Despite numerous advances in treatment utilizing the most recent protocols for surgery, radiation and chemotherapy, the cure rates and survival rates have not improved during the last 40 years, 5 year survival rate remaining approximately 55%. And also the current TNM system is inadequate to accurately classify the patients in terms of prognosis.

Thus with recently developed molecular tools the interest lies in the study of distribution of the molecular markers p53, cyclin D1 and CD44 in oral squamous cell carcinoma and their association with histopathological grading and cervical lymph node metastasis of this tumor. This will help to identify the subgroup of patients with poor prognosis who may need intense treatment strategies and also to guide treatment options thereby improving the survival rates in these patients.

REVIEW OF LITERATURE

INCIDENCE OF ORAL SCC:

More than 90% of malignant neoplasms of the oral cavity and oropharynx are squamous cell carcinomas of the lining mucosa with relatively rare neoplasms arising in minor salivary glands and soft tissues.(WHO). Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck region and accounts for over 300,000 new cancer cases worldwide every year.[15] In the US, incidence of new oral cancer estimated in the year 2009 is 35,720 as compared to the incidence for all cancers that is estimated to be 1,479,350. It is the ninth most common cancer among men in the US. Death rates are declining from 5.61 and 2 per 100,000 in males and females respectively in the year 1990 to 3.84 and 1.4 per 100,000 in the year 2005 amounting to a decrease of 31%. Although the death rates are declining the five-year overall survival rates remain around 50% over the past several decades (53% in 1974 to 60% in 2004). [16, 17] India has one of the highest incidences of Oral cancer in the world. [18] The high incidence of oral cancer and oral pre-cancerous lesions in India has long been linked with the habit of betel quid chewing incorporating tobacco. Oral cancer ranks number one among men and number three among women in India. Oral cancer constitutes 12% of all cancers in men and 8% of all cancers among women.[19] Annual incidence rate is estimated to be 64,460. However total number of cases at any given time will be 2.5 to 3 times higher than this number. It is unfortunate that so far no proper epidemiological data on this disease is

available in India. Information currently available is mostly on the basis of crude incidence rate available from three metropolitan cities covered under National cancer registry project.

AGE DISTRIBUTION:

Oral carcinoma is largely a disease of the elderly and the incidence rises sharply with age. Seventy percent of the cancer develops between 55 and 77 years of age.

Although primarily a disease of the middle and older age groups, a younger patient population presenting with oral cancers has increased alarmingly in the recent years. In this younger group, the etiological factors associated with oral cancer development remain poorly defined with proposed familial, occupational, immune deficiency and viral linked factors most often favoured.

GENDER DISTRIBUTION:

Men are affected more often than women because of heavier indulgence in both tobacco and alcohol habits in most countries; In India, the highest rates of intraoral cancer may be found in women who chew tobacco heavily.

ETIOLOGY OF OSCC:

Epidemiologic data established tobacco and alcohol use as the major causes of oral cancer.[20-22] Oral cancer risk is almost 10 times greater in individuals who smoke and drink than who do not and almost 100 times

greater in persons who smoke and drink heavily. A substantial percentage of the people with these risk behaviours, however, do not develop cancer. Other OSCC risk factors are betel quid chewing and possibly marijuana use.[23, 24] These factors, and tobacco and alcohol use, also increase the risk of oral IEN.[25] Although human papilloma virus (HPV) infection has been hypothesized for decades to play a role in the etiology of oral neoplasia, various studies have found different and contradictory frequencies of HPV DNA detection in oral mucosal lesions.[26, 27] A population-based study of 900,000 persons indicated that HPV infection is less a factor in developing OSCC than in developing nasopharyngeal or laryngeal cancer.[28] Furthermore, recent data from the largest sample size yet analyzed could not establish a link between HPV infection and the development of either regular IEN or more-aggressive verrucous oral IEN.[29] Nevertheless, HPV may be involved in some patients who develop oral neoplasia, for example, in a subset of OSCC patients without tobacco or alcohol risk factors. In addition, immune deficiency as seen in patients receiving immune suppressive therapy for organ transplant can play a role in development of oral cancers.[30] In contrast patients infected with HIV are not predisposed to oral squamous cell carcinoma.[30]

MOLECULAR PATHOGENESIS OF OSCC:

ORAL CARCINOGENESIS-A MULTISTEP PROCESS:

OSCC evolves through a multistep process of genetic, epigenetic, and metabolic changes resulting from exposure to the carcinogens discussed above.[20, 31] The clinical natural history of OSCC development usually involves normal oral mucosa changing to oral leukoplakia (or IEN) changing to OSCC. Illustrated by molecular progression models (Fig 1), oral IEN is a pathologically discernable intermediate state between normal epithelium and invasive cancer. Clinically relevant IEN has genetic or epigenetic alterations, loss of cellular control, phenotypic characteristics overlapping those of invasive cancer, and a substantial risk of biologically aggressive cancer.[32] Accumulating molecular or genetic and epigenetic, alterations within oral carcinogenesis include alterations of tumor suppressor genes such as FHIT (loss of heterozygosity [LOH] at chromosomal region 3p14), p16 (promoter hypermethylation or LOH at 9p21), and p53 (inactivation/loss or mutation at 17p), cyclin D1 overexpression (and gene amplification at 11q13), and telomerase activation.[20, 31] Tobacco may cause oral cancer, in part, via effects on p53 and the chromosomal region 3p. Altered p53 expression is associated with increased genomic instability (eg, aneuploidy) in oral IEN and may drive the acceleration in the rate of genetic alterations during oral tumorigenesis.[33] The overexpressions of cyclooxygenase-2 (COX-2) and phospho-epidermal growth factor receptor (pEGFR) also are important events in oral carcinogenesis.

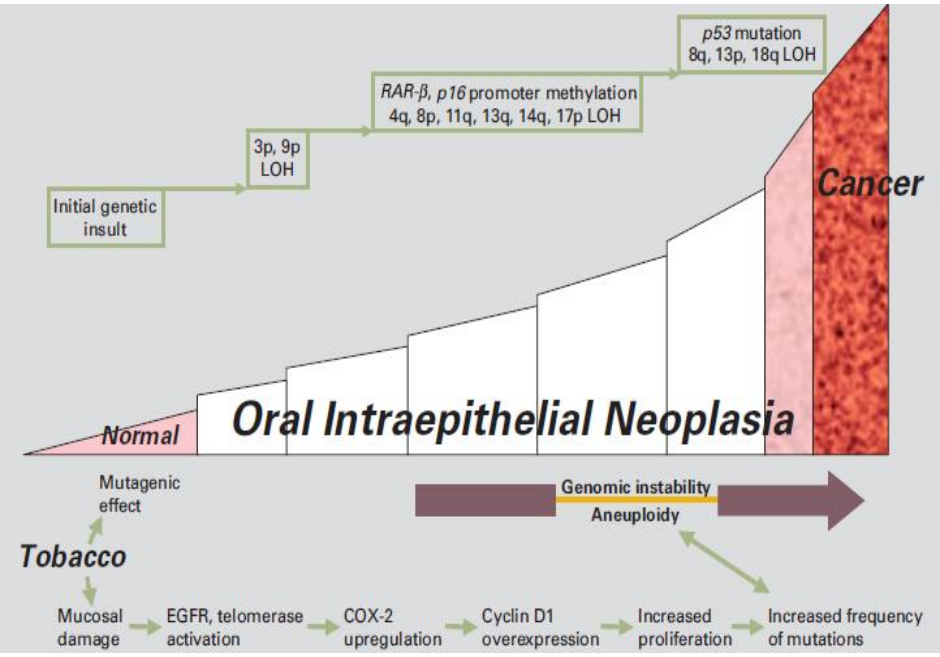

Figure 1: Molecular (genetic & epigenetic) progression model of multistep oral carcinogenesis. The white central steps of the figure represent the progression of oral intraepithelial neoplasia from leukoplakia (white patches) to erythroplakia (red patches), which can precede cancer. This process involves activation of the epidermal growth factor receptor (EGFR) and related downstream events (eg, involving cyclooxygenase-2 [COX-2] and cyclin D1) leading to dysregulated proliferation, increasing frequency of mutations causing genomic instability (and vice versa) and invasion. (LOH-loss of heterozygosity; RARβ-retinoic acid receptor-beta).

A number of studies have revealed the pivotal role played by proto-oncogenes and tumour suppressor genes in cell cycle regulation and apoptosis, indicating their aberrant expression during the course of evolution of various human cancers. The Rb pathway and the p53 pathway are two important, interconnected biochemical pathways frequently perturbed in human cancer. It

has been reported that more than 90% of oral tumours had at least one abnormality affecting either Rb or cyclin D1 or p16 and the data suggest that this pathway is a near universal target in oral carcinogenesis. After activation by cyclin D1, CDK4 or CDK6 is able to phosphorylate the Rb protein, leading to its functional inactivation and release of transcription factors necessary for entry into S phase and cell cycle progression.

p53 is reported to play a key role to ensure genomic integrity. In order to facilitate this, apoptosis should be tightly coupled to cell cycle checkpoints. In response to a variety of types of DNA damage, the p53 tumour suppressor gene product is activated and regulates a number of downstream cellular processes such as cell cycle arrest, apoptosis and DNA repair. In our study we have investigated the expression of Rb pathway protein, Cyclin D1 and p53 pathway protein, p53 in oral SCC.

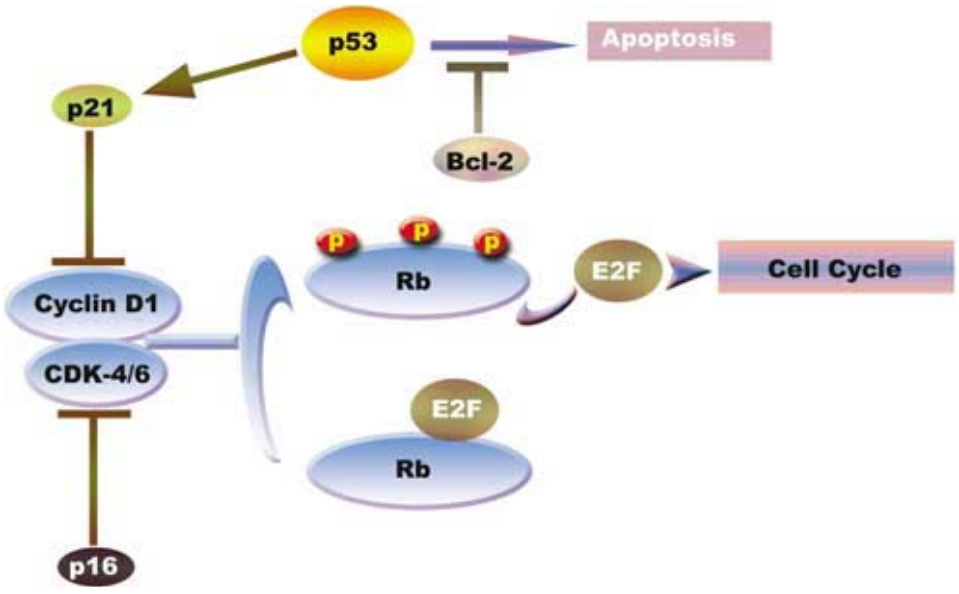

Figure 2: Flow diagram showing the role of major components of p53 and Rb pathways in cell cycle regulation.

CELL CYCLE DYSREGULATION IN CANCER:

Dysregulation of the cell cycle machinery is a fundamental hallmark of cancer progression.[34] The cellular programs of proliferation, differentiation, senescence, and apoptosis are intimately linked to the cell cycle regulatory machinery. Many of the molecular alterations that cause abnormal biologic behavior of cancer cells are based on aberrations of cell cycle regulation. For example, escape from dependence on mitogens or induction of resistance to anti-mitogens, tolerance to DNA damage, apoptosis resistance, and progression of cells with activated oncogenes and/or inactivated tumor suppressor genes through multiple checkpoints resulting in increased genomic instability—all affect and/or are affected by cell cycle regulatory proteins.[34]

The cyclins and cyclin-dependent kinases (CDKs) form the core of cell cycle regulation.[35] Expression of cyclins is cell cycle phase-dependent and is regulated transcriptionally, post-transcriptionally, and translationally/ posttranslationally. The cyclin family members interact with CDKs, and these complexes are required to pass through specific phases of the cell cycle. D-type cyclins interact with CDK4 and CDK6 and are necessary for G0/G1 transition. Cyclin E binds to CDK2 and mediates S phase entry. The cyclin A/CDK2 complex regulates passage through the S-phase. Later, in conjunction with CDC 2, cyclin A also induces the G2 phase of the cell cycle. Cyclin B1 and CDC2 trigger the molecular events associated with mitosis.[34]

CYCLIN D1 AND MITOGEN-ACTIVATED CELL CYCLE PROGRESSION:

D-type cyclins represent a link between upstream mitogenic stimuli and regulation of pRB function.[36] Human cyclin D1 was first isolated in human parathyroid adenomas as a gene rearranged by translocation to the parathyroid hormone locus at 11q13.[37] Two other human D-type cyclin genes, cyclins D2 and D3, have also been cloned. All three human D-type cyclin genes encode 33-34-kDa proteins that share an average of 57% identity over the entire coding region and 78% in the cyclin box, the region of the cyclins that interacts with CDKs. There is compelling evidence for a role of cyclin D1 in G1 phase progression in the cell cycle. Microinjection of cyclin D1 antibody or antisense cyclin D1 blocks cells from entering the S phase.[38] Overexpression of cyclin D1 accelerates progression through the G1 phase of the cell cycle and reduces the requirement of the cell for mitogens.[39] D-type cyclins and their catalytic partners, CDK4 and CDK6, play an important role in modulating the response to extracellular stimuli and are believed to be essential for passage through the G1 phase. [36]

Cyclin D1's role as an oncogene has been established by its ability to cooperate with RAS or complement a defective adenoviral E1a oncogene in cell transformation assays.[40-44] Overexpression of cyclin D1 has been reported in a variety of human tumors, including breast carcinomas, mantle cell lymphomas, and squamous cell carcinomas derived from the oral cavity, larynx, and esophagus as well as from other sites. [45] The mechanisms underlying cyclin D1 overexpression in cancer include gene amplification, chromosomal translocation, and mitogenic stimulation of gene transcription.[40] The abrogation of mitogenic pathways that might

lead to overexpression of cyclin D1 during oral carcinogenesis has been reviewed extensively elsewhere.[46] In the majority of primary oral cancers and cell lines, cyclin D1 overexpression appears independent of p16INK4a inactivation.[47] The role of cyclins D2 and D3 in oral carcinogenesis is poorly understood. Expression of antisense cyclin D1 induces apoptosis and tumor shrinkage in squamous cell carcinomas.[48] Moreover, cyclin D1 overexpression has also been linked to increased risk of occult metastases and poor prognosis in oral cancer patients.[49]

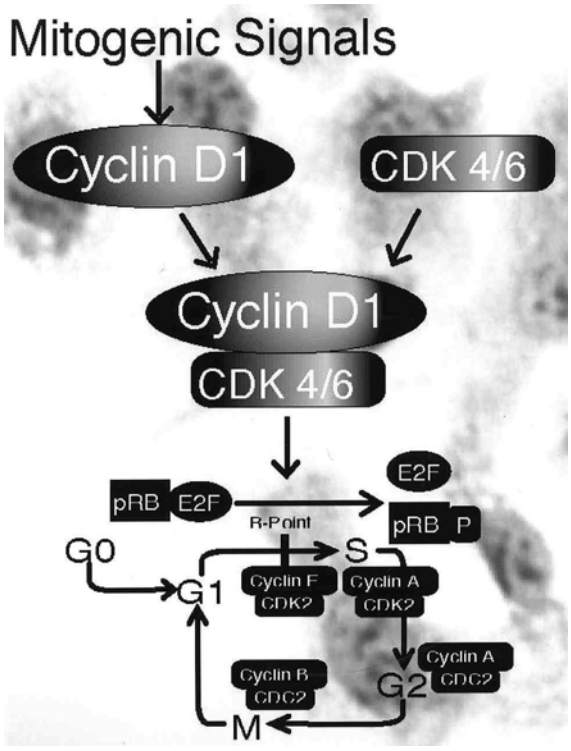

Figure 3: Mitogen Signals. Mitogen-activated G1 transition to the S phase. Mitogen stimulation leads to cyclin D1 synthesis. Together with its catalytic partners CDK4 and CDK6, cyclin D1 accelerates G1 progression by phosphorylating pRB. It is now apparent that improved treatment for OCSCC hinges on understanding the underlying dysregulation of the molecular processes in OCSCC.

THE P53 PATHWAY AND GENOMIC DAMAGE:

Genomic damage and other cellular stress signals elicit a cellular response pathway that delays or prevents cell division. The p53 tumor suppressor protein acts as a master regulator of this pathway and induces cell cycle inhibition and/or apoptosis following DNA damage. Under normal cellular growth conditions, p53 has a short half life, and the cellular steady-state levels are very low. When treated with a variety of DNA-damaging agents, such as ultraviolet or ionizing radiation and certain chemotherapeutic drugs, normal cells respond by a rapid, non-transcriptional induction of p53 as well as posttranslational modification including phosphorylation through the ATM/ATR and DNA PK family of protein kinases.[50] Depending on the circumstances, this induction of p53 causes cell cycle arrest or apoptotic cell death.[51] The p53-mediated induction of apoptosis is the subject of intense research and involves both transcriptional and non-transcriptional mechanisms.[52-57]

The induction of G1 growth arrest by p53 is mediated by the transcriptional activity of p53. Expression of p21WAF1/CIP1 mRNA and protein is strongly induced by p53 under these conditions, and p21WAF1/CIP1 can itself inhibit cell proliferation upon introduction into cultured cells.[58, 59] The ability of p21WAF1/CIP1 to inhibit many different cyclin/CDK complexes suggests that p21WAF1/CIP1 has a broad effect on cell cycle progression.[60] This p53- mediated cell cycle arrest in response to DNA damage is thought to prevent the perpetuation of genomic mutations by allowing a cell to repair DNA damage before it undergoes

a new round of DNA replication. Thus, p53 is involved in maintaining the integrity of the genome and has been referred to as the 'guardian of the human genome'.[61] Moreover, it is widely believed that the common absence of functional p53 in human tumors contributes to genomic instability, which is a hallmark of human tumors and pathologically manifested as nuclear pleomorphism.

THE P53 PATHWAY AND ABERRANT CELL PHYSIOLOGY:

The p53 and pRB tumor suppressor pathways are linked in many ways. On one hand, the ability of p53 to induce G1 growth arrest critically depends on the integrity of pRB.[34] Conversely, abnormalities in the pRB pathway are sensed by p53. Normally, E2F activity is tightly regulated by pRB. Dysregulated E2F activity, for example, caused by a mutation in the pRB pathway triggers apoptosis that is at least in part p53-mediated. Loss of p53 function is well-documented in oral cancers, and p53 mutations have been reported in over 60% of oral squamous cell carcinomas. [62] Mutation of p53 frequently induces a stabilization of the mutated protein. While little p53 is detected in normal oral epithelia and low-grade leukoplakias (mild to moderate dysplasia), p53 accumulation (indicative of p53 mutation) is more frequent in high-grade leukoplakias (severe dysplasia).

Accumulation of the mutated p53 in malignant oral epithelium has been demonstrated by several immunohistochemical studies.[63, 64] Elevated p53 in oral cancers correlates with heavy smoking but not with patient age.[65-67] However, younger patients demonstrate p53 accumulation earlier during malignant progression (Castle et al., 1999). Mutant p53 has been investigated as both a diagnostic and a therapeutic adjunct.[68-71] Last, p53 has been used as a prognostic

marker for current head and neck cancer therapy.[72-75] These studies mostly correlate the mutation of p53 with an unfavorable response to chemotherapy and radiation. However, the heterogeneity of p53 mutations renders the design of simple assays for this biomarker exceedingly difficult. [76]

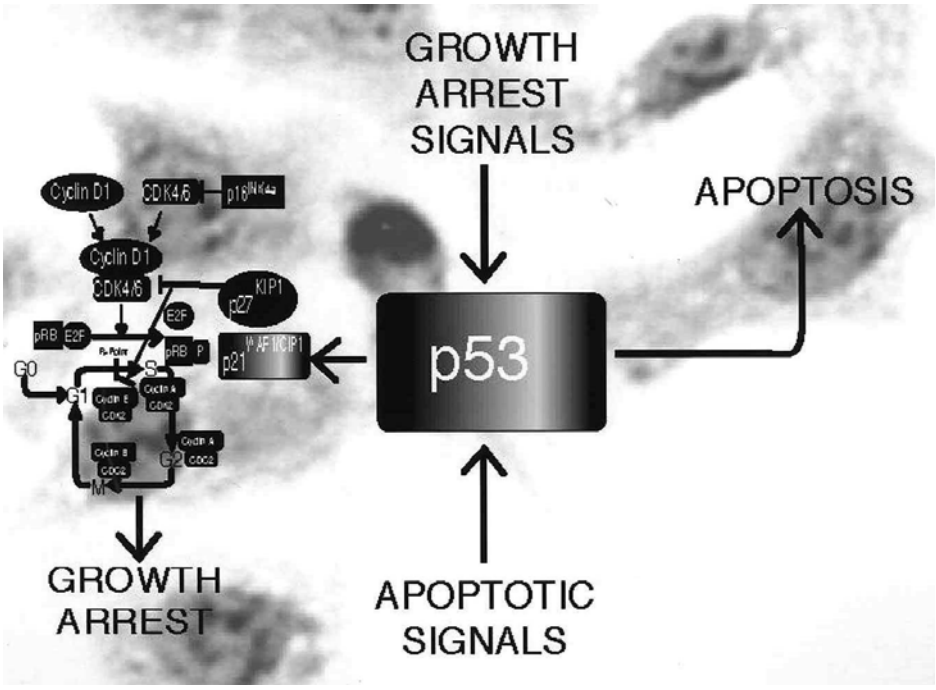

Figure 4: p53 and cell cycle regulation. Metabolic stresses (such as anoxia) and DNA damage lead to elevated p53 activity, resulting in cell cycle arrest and apoptosis.

CD44:

Besides alterations in their cell-cycle control mechanisms, tumour cells must have the ability to invade adjacent and distant tissues. Although the phenotypic changes that increase the capacity of tumour cells for invasion are not well known [9], alterations in the expression of intercellular adhesion molecules on the tumour cell surface have been implicated. [10-12]

CD44 is the major human cell surface receptor for hyaluronate and functions in a diverse range of physiological processes. CD44 may play a role in stimulating in vivo aggressiveness of tumors through hyaluronate-rich stroma.[77] Expression of CD44 has been described to correlate with metastasis formation in various tumors, although evidence in oral cavity cancers is inconclusive. The purpose of the present study was to examine CD44 expression in oral cavity cancers and to investigate its correlation with histological grading and cervical node metastasis along with cell cycle regulators p53 and Cyclin D1.

The CD44 glycoproteins are well characterized members of the hyaluronate receptor family of cell adhesion molecules. This group is defined functionally, rather than structurally, and binds to ligands of the extracellular matrix (ECM). The major ligand is hyaluronate, which is an abundant extracellular polysaccharide found in mammalian ECM, but CD44 appears to have many varied functions dependant on the extracellular structure of the protein, which can be produced in a myriad of isoforms. The wide range of functional proteins is produced from a single gene by both alternative splicing and post-translational modification.[78]

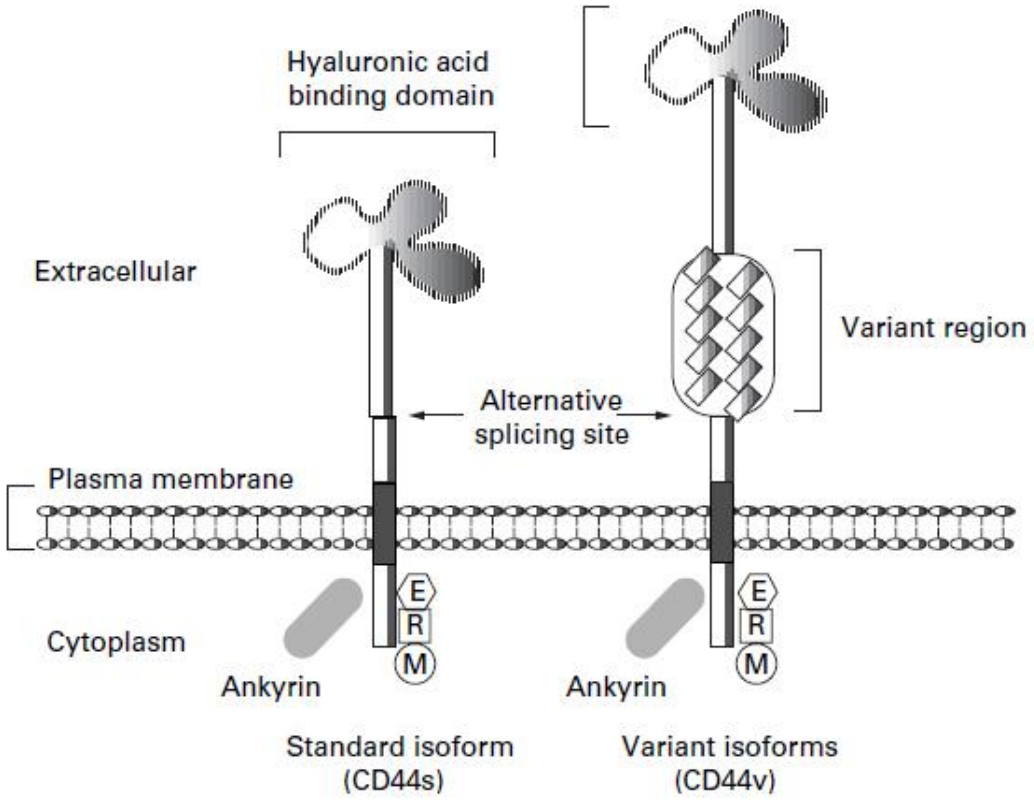

Figure 5: CD44 protein structure. The standard isoform binds its principal ligand, hyaluronic acid at the N-terminal, distal extracellular domain. The inclusion of combinations of the variant exons (v1–10) within the extracellular domain can alter the binding affinity for hyaluronic acid and confer interaction with alternative ligands. The molecule interacts with the cytoskeleton through the binding of ankyrin and the ERM family (ezrin, radixin, moesin) to the cytoplasmic domain.

CD44s, as its name implies, was first isolated on hemopoietic cells.[79] It has since been found on a wide range of tissues including the central nervous system, lung, epidermis, liver, and pancreas.[12, 80, 81]

PHYSIOLOGICAL ROLES OF CD44:

The varied structure and distribution of CD44 suggests that the molecule has a variety of functions. Known functions of CD44 are:

- Cellular adhesion (aggregation and migration)
- Hyaluronate degradation [82]
- Lymphocyte activation [83-87]
- Lymph node homing [79, 88]
- Myelopoiesis and lymphopoiesis [89-91]
- Angiogenesis [92]
- Release of cytokines [93].

ROLE OF CD44 IN TUMORIGENESIS AND METASTASIS:

CD44 functions are principally dependant on cellular adhesion in one setting or another.[94] This adhesion can lead to interaction between two different cells or between a cell and its pericellular matrix. There are many potential theories about the possible mechanisms involved with respect to the role of CD44 in tumorigenesis. CD44 expression is associated with a high rate of cell division. The proliferation status of tumour cells increases when cultured on anti-v6 antibody coated plates. CD44v6 on the cell surface is thought to crosslink with other CD44v6 molecules, initiating signals of growth promoting activity.[91] Interactions between CD44 and its ligands might induce the tumour cells to produce autocrine growth factors. These factors might be critical for tumour growth. The functions of CD44 beyond cellular adhesion

require the transmission of intracellular signals. Some of these signals are thought to occur via the cytoskeleton and might enable CD44 to signal to both the locomotory[92, 95] and mitogenic machinery of the cell.[92]

Metastatic spread requires a series of interactions between the tumour cells and the surrounding extracellular matrix and nontumour cells. These interactions will depend on cell surface determinants such as extracellular receptors for matrix and basal lamina, surface bound proteolytic enzymes, cell adhesion molecules, growth factors, and growth factor receptors.[96] The metastasising tumour cell copies the same mechanisms of normal cellular migration. From the review of the physiological functions of CD44 it can be seen that CD44 can function as a cell surface determinant for several of the roles required for metastatic spread to occur. The theoretical steps of the metastatic process are known as the metastatic cascade.[97, 98] and they consist of: (1) loss of contact with the surrounding tumour cells or neighbouring cells; (2) breakthrough of the basement membrane and penetration of vessel walls; (3) survival of shearing forces in the bloodstream/lymph stream; (4) adhesion and penetration through the vessel walls; (5) expansion into foreign tissue; (6) induction of vascularisation of tumour.

For a tumour cell to lose contact with neighbouring tumour cells, its adhesive properties must change. Changing the cell's CD44 profile could certainly achieve this. Increased expression of CD44 can enhance binding to hyaluronate and a pericellular matrix of hyaluronate might decrease the affinity of a cell for surrounding hyaluronate deficient cells by interfering with

adhesion processes, thus leading to detachment. This increased mobility is thought to be initiated by CD44 because it is linked to the cell's cytoskeleton.[92, 95] A CD44– ligand complex could mediate the mechanical force and transmit intracellular locomotory signals via the cytoskeleton. This response could lead to the cells enhanced movement along hyaluronate rich surfaces.[99] As previously discussed, CD44 has the ability to take up and degrade hyaluronate,[82] and this property could allow tumour cells to escape entrapment within hyaluronate rich environments.

Migration of any cell to the vascular or lymphatic system requires both cell adhesion molecules and cell surface enzymes. The ability of CD44 to degrade hyaluronate could also be used by the tumour cell to assist in its path through the basement membrane and vessel wall. Tumour cells that metastasize by way of the lymphatic system are thought to imitate lymphocytes, entering peripheral lymphatics and travelling to the draining lymph nodes.[86] Variants of CD44 are involved in the activation of lymphocytes and the release of cytokines.[93] CD44s is required for lymphocyte homing within the lymphatics to the high endothelial venules within lymph nodes.[79, 88] In a lymphoma animal model, CD44 monoclonal antibodies did not inhibit spleen metastases developing, whereas they did affect the incidence of metastases to the lymph nodes.[100] These observations imply that special variant isoforms of CD44 may be involved in the specific homing of tumour cells.

THE CONCEPT OF SECOND PRIMARY TUMORS IN UPPER AERODIGESTIVE TRACTS:

Local treatment of oral IEN (particularly aggressive IEN) has produced dismal results with respect to oral cancer prevention. A major probable cause of these poor results is the field process of oral carcinogenesis. The concept of ‘‘field cancerization’’ was introduced by Slaughter et al [101] more than 50 years ago and was based on the exposure of wide fields of epithelial surface within the aerodigestive tract to carcinogens such as tobacco and alcohol, thus increasing the risk of cancer development. According to the field cancerization model, multiple oral cancers arise from separate or independent cell clones. However, genetic analyses indicate that subsequent cancers distant from the original tumor also may derive from the spread of the original clone.[102] It is hypothesized that an oral epithelial stem cell initially acquires a genetic alteration and parents a clonal unit consisting of itself and daughter cells with the same DNA alteration. Next, a lesion patch progresses into an expanding field as a result of additional genetic alterations. This mucosal field replaces the normal epithelium and may be visible as oral IEN. Ultimately, clonal selection leads to the development of carcinoma within this field of IEN cells. This model entails the important clinical implication that fields often remaining after surgery of the primary IEN/tumor may lead to new cancers, presently designated as second primary tumors (SPTs) or local recurrences.

GENERAL PATHOLOGIC FEATURES OF ORAL SCC:

Oral epithelial dysplasia: Features characteristic of oral epithelial dyaplasia are the classic cytological abnormalities associated with most epithelial atypias.

Microscopic features of oral epithelial dysplasia:

- An increased nuclear cytoplasmic ratio.
- Sharp angled rete processes.
- Loss of cell polarity.
- Cellular pleomorphism.
- Nuclear pleomorphism.
- Enlarged nucleoli.
- Reduction of cellular cohesion.
- Individual spinous layer cell keratinization.
- Increased number of mitotic figures.
- Presence of mitotic figures in the superficial half of the epithelium.
- Basal cell layer hyperplasia.
- Loss of basal cell polarity.

CARCINOMA IN SITU:

The diagnosis of carcinoma in situ of the oral mucosa is based on rigid histological criteria. Classically a carcinoma in situ sample should show all the atypical cytologic criteria necessary for a malignant diagnosis, but these atypical changes must be confined to the epithelial layer. With carcinoma in

situ, one must identify an intact basement membrane and top to bottom epithelial dysplasia. Lesions that are diagnosed as carcinoma in situ may appear red, white, blue or black clinically and on occasion they may present as a tumor mass.

PATHOLOGICAL FEATURES OF INVASIVE SCC:

GROSS:

Grossly squamous cell carcinoma of the oral cavity can present as an ulcer, an alteration of mucosal color or a tumor mass. Ulcerative lesions usually have a crateriform appearance with roled elevated borders that are firm because of the infiltration of tumor along the margins. The cut section usually has a grey white glistening appearance with little tendency to bulge beyond the cut margins.

MICROSCOPY:

A proliferation of sheets, nests, chords and neoplastic islands of epithelium that penetrate into the supporting connecting tissue lamina propria and submucosa characterize squamous cell carcinoma. The neoplasm is usually identified histologically as been well differentiated, moderately differentiated, poorly differentiated or undifferentiated (non-keratinizing). Tumors are generally graded as grades I to IV, in which grade I tumors closely resemble the tissue of origin and grade IV tumors demonstrate very few features that resemble tissue of squamous epithelial origin.

The neoplastic cells of well differentiated squamous carcinomas bear a striking similarity to the cells of normal squamous epithelium. The cells are

generally large with vescicular to oval nuclei and eosinophillic cytoplasm, intracellular bridging is usually easily discernible, and the degree of nuclear hyperchromatism and bizarre mitotic activity is minimal. Keratin pearl formation is usually quite prominent in well differentiated squamous cell carcinoma, and individual cell keratinization tends to be a hallmark of this disease. As the tumor becomes less differentiated, although the tumor cells resemble normal squamous epithelial cells, hyperchromatism, pleomorphism and loss of attachment of cells are more prominent. The frequency of atypical mitosis is increased and the frequency of individual cell keratinization and keratin pearl formation is decreased. In poorly differentiated squamous cell carcinomas, there is very little evidence that the tumor is of squamous origin and there is significant pleomorphism and atypical mitosis. Undifferentiated squamous cell carcinomas have little if any resemblance to a neoplasm of squamous epithelium with the cells resemling histiocytes, atypical lymphocytes or spindle fibroblasts. Electron microscopic evaluation and immunohistochemical staining for keratin may be the only method of documenting that the tumor is of squamous epithelial origin.

VERRUCOUS CARCINOMA:

Verrucous carcinoma is a variant of well differentiated SCC endowed with enough clinical, pathologic, and behavioral peculiarities to justify it being regarded as a specific tumor entity.[103-106] Within the oral cavity the most common sites are buccal mucosa and lower gingiva.[107] Most patients are elderly males and there is a close connection with the use of tobacco especially chewing or snuff dipping.

Grossly it presents as a large fungating soft papillary growth that tends to become infected and slowly invades contiguous structures. It may grow through the soft tissues of the cheek, penetrate into the mandible or maxilla, and invade perineurial spaces. Regional lymphnode metastasis is exceedingly rare, and distant metastases have not been reported. Microscopically verrucous carcinoma shows hyperkeratosis, acanthosis, benign appearing papillomatosis, and most importantly swollen and voluminous rete pegs that extend into the deeper tissues.[108] The most important differential feature with a case of well differentiated squamous cell carcinoma is a good cytological differentiation throughout the tumor.

OTHER MICROSCOPIC SUBTYPES:

Adenoid (Pseudo glandular) squamous cell carcinoma: This tumor exhibits a pseudo-glandular or alveolar appearance because of acantholysis.

Adenosquamous carcinoma: In contrast to the above, this rare variant shows areas of squamous differentiation mixed with others having true glandular differentiation.[109, 110]

Basaloid squamous cell carcinoma: This is an aggressive variant of squamous cell carcinoma that has a predilection for the upper aerodigestive tract. Microscopically areas with obvious squamous differentiation are admixed with solid tumor islands that exhibit peripheral palisading on a thick basement membrane which is one of the striking attributes of this tumor.[111]

Spindle cell carcinoma: In this tumor the sarcoma like formation blends with areas of obvious squamous cell carcinoma or is associated with squamous

cell carcinomas elsewhere in the oral cavity or represents the recurrence of an original squamous cell carcinoma.[112-115]

Papillary squamous cell carcinoma: The histological feature of this type includes the presence of a papillary display of fibrovascular cores lined by markedly dysplastic squamous epithelium.

Lymphoepithelioma: This is a histological variant of SCC in which there is intermingling of undifferentiated carcinoma cells with prominent lymphoid stroma.

LOCAL AND DISTANT METASTASIS:

SCC of the upper aerodigestive tract (UADT-SCC) predominantly metastasizes to the lymphnodes of the neck, the site of the involved nodes being dependant on the localization of the primary tumor.[116, 117] The adverse influence of metastatic neck node deposits on patient survival is firmly established, the prognosis being diminished roughly by half if lymphnode metastases are present at presentation or during follow-up.[118, 119] Prognosis further worsens if the tumor spreads beyond the lymphnode into the soft tissues of the neck; this growth pattern is known as extracapsular spread.

Neck node disease also correlates with increased risk for development of distant metastasis. Patients with disease in the neck had twice as many distant metastasis as those without (13.6% vs 6.9%), whereas the presence of extranodal spread meant a 3-fold increase in the incidence of distant metastasis, compared with patients without this feature. The occurrence of distant metastasis of UADT-SCC has been proved to predominantly occur in the lungs.

Thus, the prognostic significance of neck node disease justifies a very meticulous examination of neck dissection specimens, as a high incidence of micrometastasis (<3mm) has been found in patients without clinically manifest neck disease. Therefore, one should realize that, although pretreatment evaluation of nodal status in many institutions is based on palpation, depending on palpation for detection or exclusion of nodal involvement has proven unreliable; nevertheless, it remains part of the initial staging.

WHO CLASSIFICATION OF TUMORS OF ORAL CAVITY AND OROPHARYNX

Malignant epithelial tumours:

Squamous cell carcinoma

- Verrucous carcinoma
- Basaloid squamous cell carcinoma
- Papillary squamous cell carcinoma
- Spindle cell carcinoma
- Acantholytic squamous cell carcinoma
- Adenosquamous carcinoma
- Carcinoma cuniculatum

Lymphoepithelial carcinoma

Epithelial precursor lesions

Benign epithelial tumours

Papillomas

- Squamous cell papilloma and verruca vulgaris
- Condyloma acuminatum

- Focal epithelial hyperplasia

Granular cell tumour

Keratoacanthoma

Salivary gland tumours

Salivary gland carcinomas

- Acinic cell carcinoma
- Mucoepidermoid carcinoma
- Adenoid cystic carcinoma
- Polymorphous low-grade adenocarcinoma
- Basal cell adenocarcinoma
- Epithelial-myoepithelial carcinoma
- Clear cell carcinoma, not otherwise specified
- Cystadenocarcinoma
- Mucinous adenocarcinoma
- Oncocytic carcinoma
- Salivary duct ca rcinoma
- Myoepithelial carc inoma
- Carcinoma ex pleomorphic adenoma

Salivary gland adenomas

- Pleomorphic adenoma
- Myoepithelioma
- Basal cell adenoma
- Canalicular adenoma

Duct papilloma

Cystadenoma

Soft tissue tumours

Kaposi sarcoma

Lymphangioma

Ectomesenchymal chondromyxoid tumour

Focal oral mucinosis

Congenital granular cell epulis

Haematolymphoid tumours

Diffuse large B-celilymphoma (DlBCl)

Mantle cell lymphoma

Follicular lymphoma

Extranodal marginal zone B-cell lymphoma of MALT type

Burkitt lymphoma

T-cell lymphoma (including anaplastic large cell lymphoma

Extramedullary plasmacytoma

Langerhans cell histiocytosis

Extramedullary myeloid sarcoma

Follicular dendritic cell sarcoma I tumour

Mucosal malignant melanoma

Secondary tumours

TNM CLASSIFICATION OF CARCINOMAS OF ORAL CAVITY AND OROPHARYNX:

TNM classification of carcinomas of the lip and oral cavity [1,2]

T – Primary tumour
- TX Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- Tis Carcinoma in situ
- T1 Tumour 2 cm or less in greatest dimension
- T2 Tumour more than 2 cm but not more than 4 cm in greatest dimension
- T3 Tumour more than 4 cm in greatest dimension
- T4a (lip) Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)
- T4a (oral cavity) Tumour invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
- T4b (lip and oral cavity) Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery

Note: Superficial erosion alone of bone/tooth socket by gingival primary is not sufficient to classify a tumour as T4.

N – Regional lymph nodes##
- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in great est dimension
- N2 Metastasis as specified in N2a, 2b, 2c below
- N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
- N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
- N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
- N3 Metastasis in a lymph node more than 6 cm in greatest dimension

Note: Midline nodes are considered ipsilateral nodes.

M – Distant metastasis
- MX Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis

Stage grouping

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2	N1	M0
	T3	N0, N1	M0
Stage IVA	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

The regional lymph nodes are the cervical nodes.

TNM classification of carcinomas of the oropharynx [1,2]

T – Primary tumour
- TX Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- Tis Carcinoma in situ
- T1 Tumour 2 cm or less in greatest dimension
- T2 Tumour more than 2 cm but not more than 4 cm in greatest dimension
- T3 Tumour more than 4 cm in greatest dimension
- T4a Tumour invades any of the following: larynx, deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), medial pterygoid, hard palate, and mandible
- T4b Tumour invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or encases the carotid artery

N – Regional lymph nodes##
- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in great est dimension
- N2 Metastasis as specified in N2a, 2b, 2c below
- N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
- N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
- N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
- N3 Metastasis in a lymph node more than 6 cm in greatest dimension

Note: Midline nodes are considered ipsilateral nodes.

M – Distant metastasis
- MX Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis

Stage grouping

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2	N1	M0
	T3	N0, N1	M0
Stage IVA	T1,T2,T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

The regional lymph nodes are the cervical nodes.

[1] (947,2418).
[2] A help desk for specific questions about the TNM classification is available at www.uicc.org/index.php?id=508 .

PROGNOSTIC AND PREDICTIVE FACTORS:

Tumor size and nodal status are the most significant prognostic factors. [120] Histological grade correlates poorly with patient outcome.[121, 122] The value of grading improves when only deeply invasive margins of the tumor are evaluated.[123, 124] Tumors invading with pushing borders are less aggressive than tumors with a non-cohesive front showing diffuse spread with tiny strands or single cells.[125-127] Major risk factors that adversely influence prognosis are 2 or more positive regional nodes, extracapsular extension of nodal disease, or positive margins of resection.[128] Other important histological features associated with poor prognosis are tumor thickness and vascular invasion. Molecular markers with unequivocal prognostic and/or predictive significance have not been identified.[129-131]

FUTURE DIRECTIONS AND CLINICAL APPLICATIONS:

The oral cancer problem primarily involves the understanding, diagnosis, and treatment of squamous cell carcinoma of the oral cavity.[132] While recent advances have reduced the morbidity of oral cancer, the five-year survival rate for these patients has remained largely unchanged at ~ 50% for the last 30 years, because early stages of the disease are associated with minimal signs and symptoms, and advanced stages generally respond poorly to current cancer therapies.[132] The ability to map the signature cell cycle defects in human oral cancer is of value not only for the biological understanding of the disease but more importantly toward the translational utilization of this information for early diagnosis and biology-

based therapy. Many of the cell cycle regulators reviewed have been associated as biologic predictors of oral cancer behavior. In the future, our ability to profile, comprehensively, the gene expression differences among normal, pre-malignant, and tumor cells from the same patient will allow us better to index the consistently altered cell cycle defects in human oral cancer.[133]

Development of new animal models will advance our understanding of the functional consequences of these cell cycle defects. Understanding the identity and function of oral cancer cell cycle defects will provide novel biological/genomic parameters for patient outcome monitoring and treatment therapy decisions and options. Two new head and neck cancer therapeutic approaches under investigation are cell-cycle-based. ONYX-015 is an E1B attenuated adenovirus that is believed to replicate selectively in p53 mutant cells, therefore sparing normal/wild-type p53 cells. However, other studies fail to correlate mutant p53 and viral replication.[134] When administered intratumorally to patients with recurrent head and neck cancer, ONYX-015 produced tumor necrosis in four of five patients with mutant p53.[135] ONYX-015 treatments, which were easily administered and well-tolerated, may serve as an adjunct to current chemotherapeutic approaches to head and neck cancer patients. Recently, a phase II trial on recurrent head and neck cancers was completed, demonstrating improved response to intratumoral ONYX-015 injection with cisplatin and 5-fluorouracil vs. either viral treatment or chemotherapy alone.[136] Flavopiridol is a novel cyclin-dependent kinase inhibitor

that has been demonstrated to have anti-neoplastic properties.[137] Recently, flavopiridol has been shown to suppress head and neck carcinoma growth by inducing apoptosis.[138] Exposure of malignant oral keratinocytes to flavopiridol diminished CDC2 and CDK2 activity, as well as reduced cyclin D1 expression. Certainly, understanding the mechanisms of cell cycle dysregulation of the oral keratinocyte during oral carcinogenesis will serve as an adjunct to present diagnostic and therapeutic options and provide the basis for novel strategies in the future.

MATERIALS AND METHODS

STUDY CHARACTERISTICS:

Twenty five patients with in situ and invasive oral SCC and its variant verrucous carcinoma who attended the out-patient department of Coimbatore Medical College Hospital between April 2008 and March 2009 were randomly included in our study. The immuno expression of the molecular markers (cyclin D1, p53, and CD44) were studied in their tissue sections and correlated with the degree of differentiation of the tumor and its association with cervical lymph node metastasis was analyzed.

PATIENT CHARACTERISTICS:

Only those patients with newly diagnosed oral SCC were included in the study. Patients who presented with regional lymph node involvement were also included in the study. Patients with distant metastasis were excluded by using X-ray of the chest, ultrasound of the abdomen and CT scan of the brain. Those patients with oral SCC who had history of recurrence of the tumor, prior radiotherapy or surgery for the tumor were excluded from the study. Patients suffering from other primary malignancies and systemic illness were also excluded from the study.

SAMPLES:

In all the patients the primary tumor was confined to one of the following anatomical locations within the oral cavity including buccal mucosa, tongue, lip, alveolar margin, retromolar trigone, tonsil, floor of mouth and

palate. So the total of 25 patients yielded 25 samples that comprised of 23 incisional biopsies obtained at the time of initial diagnosis and 2 wide excision specimens from patients curatively resected for cancer. Split up of the specimens showed their origin to be 6 each from buccal mucosa and tongue, 3 each from lip and alveolar margin, 2 each from retromolar trigone, tonsil, and palate and one from floor of mouth.

For histopathological and immunohistochemical studies the tumor samples were fixed in 10% buffered formalin and then embedded in paraffin. The diagnosis was confirmed by routine histopathological examination using hematoxylin and eosin stain. For immunohistochemical studies, 4 micrometer thick tissue sections were taken in specially coated slides using chrome-alum and gelatin. The tissue sections included not only tumor lesions but also the adjacent non-tumorous oral epithelium in few cases that served as internal controls for immunohistochemistry.

In patients with enlarged cervical nodes, fine needle aspiration was done and the cytology smear was examined to confirm the presence of metastatic deposit.

IMMUNOHISTOCHEMISTRY:

PRINCIPLE:

The demonstration of antigens in tissues and cells by immunostaining is a 2-step process involving first, the binding of an antibody to the antigen of interest and second, the detection and visualization of bound antibody by one of a variety of enzyme chromogenic system. In our study we have used

"The Super Sensitive Polymer-HRP Detection System" that is based on a non-biotin polymeric technology that makes use of 2 major components: Super Enhancer and a Poly-HRP reagent. As the system is not based on the Biotin-Avidin system the problems associated with endogenous biotin are completely eliminated. In this technique a large number of peroxidase enzyme molecules are bound to a secondary antibody via the dextran backbone. This was done to increase the sensitivity.

SIMPLE PROTOCOL :

1. Application of primary antibody
2. Application of enzyme labeled polymer
3. Application of the substrate chromogen

EVALUATION OF IMMUNOHISTOCHEMICAL STAINING:

The most representative tumor areas were selected for scoring the immunostaining pattern. The scoring was done using light microscopy. The following criteria were used to study the distribution and intensity of positive tumor cell staining. Nuclear coloration was considered as a positive reaction for p53 and Cyclin D1 and cell membrane staining was considered as a positive reaction for CD44.

P53 AND CYCLIN D1 SCORING SYSTEM: [139]

Distribution: Absent tumor cell staining was scored as 0, <10% of positive tumor cells staining were scored as 1, 10% to 50% of cells staining

were scored as 2, 50% to 90% of cells staining were scored as 3, and >90% cells staining were scored as 4.

Intensity: Absent staining in tumor cells was scored as 0, equivocal was scored as 1, clearly positive was scored as 2, and strong positive staining was scored as 3.

The results for intensity and distribution were summed and a "score" was assigned from 0 to 7. Over expression for each of these antibodies was assigned when a score of ≥ 4 was obtained.

CD44 SCORING SYSTEM:

The degree of positive staining for CD44 antibody was evaluated by a well-established semiquantative scoring on a scale of 1 to 4 for intensity (I) such as none, mild, moderate and strong, and for distribution (D) such as none, focal, patchy and diffuse (7). Tissues with I x D less than or equal to four were considered weakly positive and those with I x D greater than four were designated strongly positive.

OBSERVATION AND RESULTS

Table I: Demographics

Parameter	Value
Age (Mean ± SD)	57.08 ± 11.17 (40-80) YEARS
Sex (Male : Female)	13:12 (52/48)%
Alcohol use	40%
Tobacco use	92%
Diabetes	20%
Hypertension	24%
Radiation exposure	0%
Family history	0%
Immune defects	0%
Occupational exposure	0%

Table I shows the demographic characteristics of our study population. The mean age of the patients was 57.08 ± 11.17 years and the youngest patient was 40 years and eldest was 80 years old. Almost half of the patients (48%) were females. Almost all (92%) patients were tobacco users in the form of smoking, chewing or snuffing. Among them 9 (39%) were smokers, 12 (52%) were chewers, and 2 (8%) were snuffers. Among males (13) 9 (69%) were smokers, one was a chewer, and 2 were snuffers. Among females almost all of them (92%) were chewers. Forty percent of the patients were alcoholics. Five patients (20%) were diabetics and six (24%) were hypertensives. No patients had other risk factors for oral carcinoma like exposure to radiation, family history of cancer, immune deficiency or occupational exposure to carcinogens.

Table II: Clinical and Histopathological Characteristics

S. NO	AGE	SEX	SPE[α]	SITE	EXPOSURE		HISTOLOGY[β]	GRADE[γ]	ADJ EPI[ε]	NODAL STATUS[ζ]
					TOBACCO	ALCOHOL				
1	46	M	WB	BUCCAL MUCOSA	SMOKER	PRESENT	VC	WDv	+	NAP
2	60	M	WB	ALVEOLAR MARGIN	SMOKER	PRESENT	In SCC	MD	-	-
3	50	F	WB	RETROMOLAR TRIGONE	CHEWER	ABSENT	In SCC	MD	+	+
4	50	M	WB	FLOOR OF MOUTH	SMOKER	ABSENT	In SCC	MD	+	+
5	65	M	WB	TONSIL	SMOKER	PRESENT	INSITU SCC	NAP	+	NAP
6	70	M	WB	TONGUE	SMOKER	PRESENT	In SCC	MD	-	-
7	68	M	WB	PALATE	SNUFFER	PRESENT	In SCC	MD	+	+
8	80	F	WB	BUCCAL MUCOSA	CHEWER	ABSENT	INSITU SCC	NAP	+	NAP
9	40	F	WB	TONGUE	CHEWER	ABSENT	In SCC	MD	+	+
10	47	F	WEB	TONGUE	CHEWER	ABSENT	In SCC	WD	+	+
11	70	M	WB	BUCCAL MUCOSA	SNUFFER	ABSENT	In SCC	WD	+	+
12	NA	M	WB	BUCCAL MUCOSA	SMOKER	PRESENT	In SCC	MD	+	-
13	52	F	WB	PALATE	CHEWER	ABSENT	In SCC	MD	-	-
14	64	F	WB	ALVEOLAR MARGIN	CHEWER	ABSENT	In SCC	WD	-	-
15	67	M	WB	TONGUE	CHEWER	PRESENT	In SCC	WD	+	-

Table II. Contd..

S. NO	AGE	SEX	SPE[α]	SITE	EXPOSURE		HISTOLOGY[β]	GRADE[γ]	ADJ EPI[ε]	NODAL STATUS[ζ]
					TOBACCO	ALCOHOL				
16	40	F	WB	LIP	NONE	ABSENT	VC	WDv	+	NAP
17	43	M	WB	BUCCAL MUCOSA	NONE	PRESENT	In SCC	MD	+	-
18	53	M	WB	TONGUE	SMOKER	PRESENT	In SCC	WD	+	+
19	60	F	WB	LIP	CHEWER	ABSENT	In SCC	MD	+	-
20	70	F	WB	TONGUE	CHEWER	ABSENT	In SCC	WD	-	-
21	70	F	WEB	TONGUE	CHEWER	ABSENT	VC	WDv	+	NAP
22	50	F	WB	LIP	CHEWER	ABSENT	VC	WDv	+	NAP
23	58	M	WB	TONSIL	SMOKER	ABSENT	In SCC	PD	+	-
24	45	M	WB	RETROMOLAR TRIGONE	SMOKER	PRESENT	In SCC	PD	-	-
25	46	M	WB	ALVEOLAR MARGIN	CHEWER	ABSENT	In SCC	WD	-	-

α:WB- Wedge Biopsy, WEB- Wide Excision Biopsy. β: VC- Verrucous Carcinoma, In SCC-Invasive Squamous Cell Carcinoma, INSITU SCC-Insitu Squamous Cell Carcinoma. γ:WD- Well Differentiated, WDv- Variant of Well Differentiated, MD- Moderately Differentiated, PD- Poorly Differentiated. ε: - - Absent, +- Present. ζ: NAP- Not Applicable,- -Absent, +- Present.

Table II shows the clinical and histopathological characteristics of the individual patients.

Table III: Nature of specimen

Sl.No	Nature of the Specimen	No of Patients (Percentage)
1	**Incisional biopsy**	23 (92%)
2	**Wide excision biopsy**	2 (8%)

Table III shows the type of specimens obtained for our study. The total of 25 patients yielded 25 samples among which only 2 were wide excision biopsies that were obtained during the curative resection of the tumor. All the rest were specimens obtained at the time of incisional biopsies done for the initial diagnosis of the lesion.

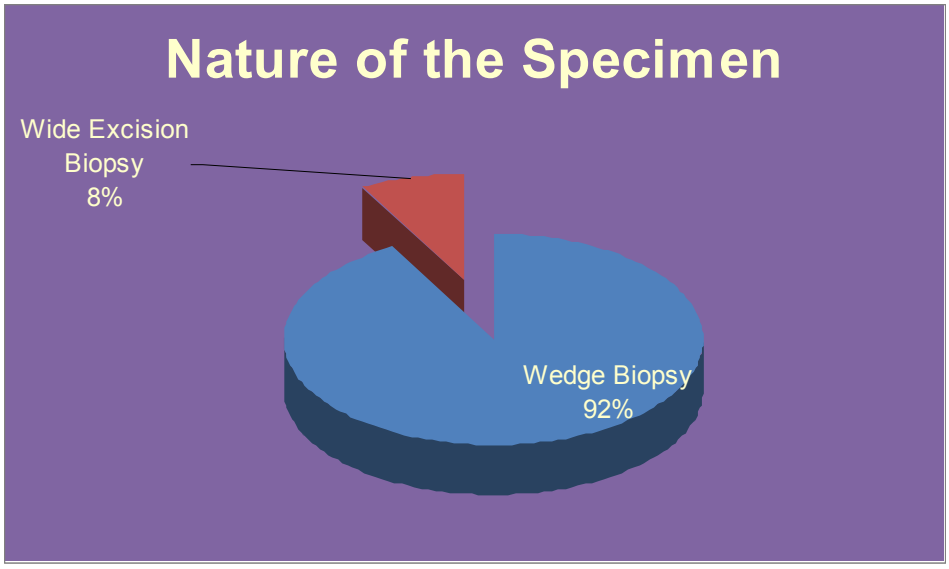

Figure 6: Pie diagram of the nature of the specimen

Table IV: Distribution of the tumor within the oral cavity

Sl.No	SITE OF THE TUMOR	Number of Patients	Percentage
1	TONGUE	6	24
2	BUCCAL MUCOSA	6	24
3	LIP	3	12
4	ALVEOLAR MARGIN	3	12
5	TONSIL	2	8
6	RETROMOLAR TRIGONE	2	8
7	PALATE	2	8
8	FLOOR OF THE MOUTH	1	4

Table IV shows the distribution of the tumor site within the oral cavity. Split up of the specimens showed their origin to be 6 each from buccal mucosa and tongue, 3 each from lip and alveolar margin, 2 each from retromolar trigone, tonsil, and palate and one from floor of mouth.

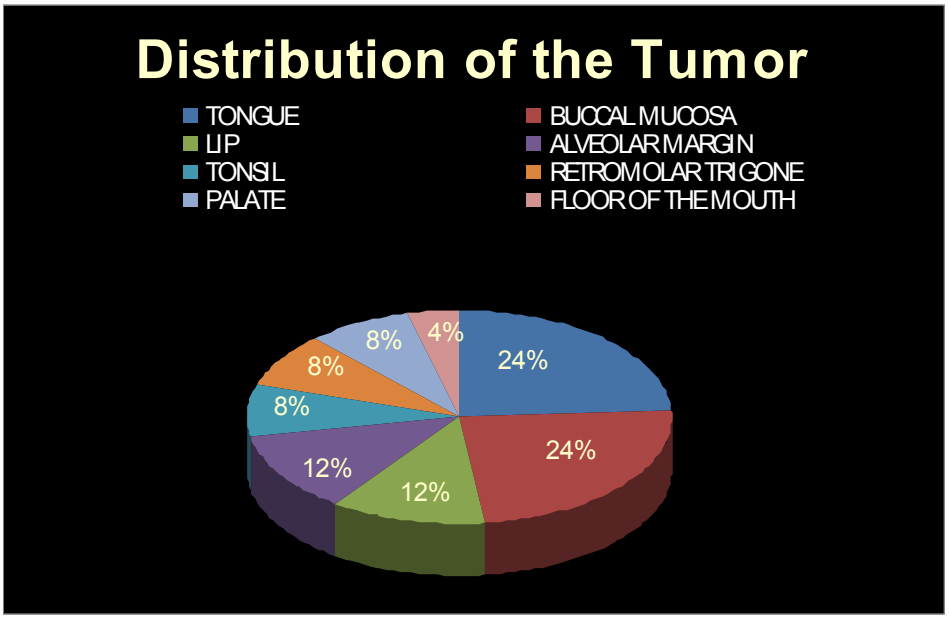

Figure 7: Pie chart depicting the distribution of the tumor

Table V: Histopathological grading and typing

Histological Type	Grade	Number of Cases
Carcinoma Insitu	-	2
Invasive Carcinoma	Well Differentiated	7
	Moderately Differentiated	10
	Poorly Differentiated	2
Verrucous Carcinoma	Variant of well differentiated carcinoma	4

Table V shows the distribution of the tumor by their different grades and types. Three forths (19) of the tumors were invasive SCC. Insitu and verrucous carcinoma contributed to 8% and 16% of the tumors respectively. Among the invasive SCC, half the tumors were moderately differentiated and were closely followed by the well differentiated SCC amounting to 37%. Poorly differentiated SCC contributed to only 10% of the invasive SCC in our study.

Table VI: Metastasis to regional lymph nodes in Invasive OSCC (n=19)

Nodal Metastasis	Number of Cases	Percentage
Positive	7	37
Negative	12	63

Table VI shows the details of the metastatic regional lymph node involvement in patients with invasive SCC. Seven (37%) of the 19 patients had regional cervical lymph node enlargement. According to the protocol these patients underwent fine needle aspiration (FNA) and the presence of metastatic deposits from the primary tumor was confirmed.

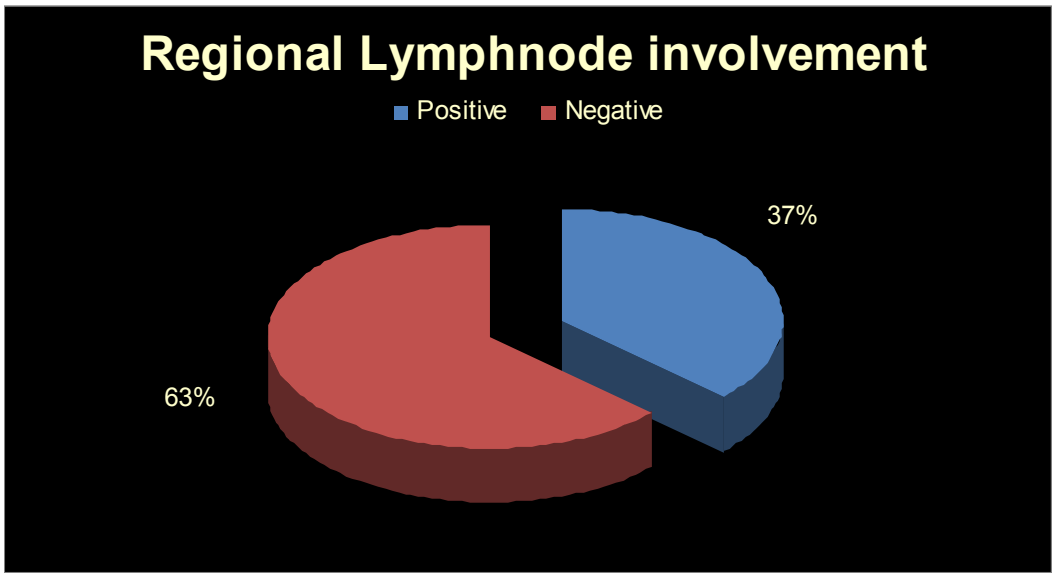

Figure 8: Pie diagram showing the regional lymphnode involvement

PATTERNS OF IMMUNOSTAINING OF THE MOLECULAR MARKERS:

Immunostaining for p53 and Cyclin D1 primarily demonstrated a nuclear staining pattern. Staining for CD44 primarily demonstrated a membrane pattern of staining with a rare component of cytoplasmic staining and no nuclear staining. Immuno study in our work was based only on membrane staining. Lymphocytes and inflammatory cells stained intensely for CD44 but were excluded from analysis. Figure 9 to 11 shows the immunostaining patterns of the molecular markers studied.

Table VII: Status of the adjacent epithelium

Status of the adjacent epithelium		No of cases
Absent		7
Present	**Normal/Hyperplastic**	15
	Dysplastic	3

Table VII shows the details of the adjacent non-tumoral epithelium. Adjacent epithelium was present only in 18 (72%) of the 25 cases studied. Among them 3 (17%) showed mild to moderate dysplasia and the remaining 15 (83%) of the specimens showed normal or hyperplastic adjacent epithelium.

Table VIII: Expression of Molecular Markers in the Adjacent Epithelium

S.NO	HPE NO	ADJ EPI	P53		CYCLIN D1		CD44	
			EXP	INT	EXP	INT	EXP	INT
1	1023/08	Present	1	4	3	3	3	3
2	108/09	Absent	NAP	NAP	NAP	NAP	NAP	NAP
3	119/09	Present	3	3	2	2	3	3
4	120/09	Dysplastic	4	4	4	4	4	4
5	1369/08	Absent	NAP	NAP	NAP	NAP	NAP	NAP
6	1601/09	Present	1	3	2	4	3	4
7	1604/09	Absent	NAP	NAP	NAP	NAP	NAP	NAP
8	1608/08	Present	3	4	3	4	3	3
9	1943/08	Absent	NAP	NAP	NAP	NAP	NAP	NAP
10	2328/08	Present	3	3	3	4	3	1
11	2348/08	Dysplastic	4	4	4	4	4	4
12	2379/08	Dysplastic	4	4	4	4	4	4
13	2525/08	Present	3	3	2	4	3	3
14	2581/08	Present	3	3	2	4	3	4
15	2717/08	Present	1	2	2	3	3	3
16	2718/08	Absent	NAP	NAP	NAP	NAP	NAP	NAP
17	2765/08	Absent	NAP	NAP	NAP	NAP	NAP	NAP
18	2798/08	Present	1	2	2	3	3	3
19	2817/08	Present	1	3	2	3	1	4
20	2827/08	Present	1	4	2	4	3	2
21	644/08	Present	3	4	2	4	3	3
22	66/09	Present	1	4	2	4	3	3
23	763/08	Absent	NAP	NAP	NAP	NAP	NAP	NAP
24	857/08	Present	1	4	2	4	3	4
25	926/08	Present	1	4	3	4	3	1

EXP: Expression (Scoring: 1-Basal layer; 2-Suprabasal layer; 3- Both basal and suprabasal layers; 4-Entire dysplastic area). INT: Intensity (Scoring: 1-Weak; 2-Mild; 3-Moderate; 4-Strong). NAP: Not Applicable.

Table VIII shows the expression of molecular markers in the adjacent epithelium.

In the sections that had normal or hyperplastic adjacent epithelium the p53 staining was expressed predominantly in the basal layer in 9 cases and both basal and suprabasal layers in 6 cases and the intensity was variable. The cyclin D1 staining was predominantly suprabasal in 11 cases and both basal and suprabasal in the remaining 4 cases. The CD44 staining was present in both the basal and suprabasal layer in all of them except one that had the staining restricted to the basal layer alone. The intensity of the staining for all the three molecular markers was predominantly moderate or strong.

In the three sections that showed mild to moderately dysplastic adjacent epithelium the intensity of the staining for all the 3 molecular markers was strong. The expression was found to be present involving the entire dysplastic area.

Table IX: Expression of molecular markers in insitu oral cancer

SITE	P53				CYCLIN D1				CD44			
	EXP	EXT	INT	TS	EXP	EXT	INT	TS	EXP	EXT	INT	TS
TONSIL	+	4	3	7	+	4	3	7	+	4	4	16
BUCCAL MUCOSA	+	4	3	7	+	1	3	4	+	2	3	6

EXP- Expression; EXT-Extent; INT-Intensity; TS- Total Score

Table IX shows the expression and scoring of immunostaining of the various molecular markers in cases of in situ oral SCC. All the 3 molecular markers were over expressed in both the cases.

Table X: Distribution of molecular markers in patients with Oral SCC

S.NO	HPE No	P53				Cyclin D1				CD 44			
		Exp	Ext	Int	TS	Exp	Ext	Int	TS	Exp	Ext	Int	TS
1	108/09	+	3	3	6	+	2	3	5	-	1	1	1
2	119/09	+	3	2	5	+	3	2	5	-	1	1	1
3	120/09	+	3	3	6	+	1	3	4	+	2	2	4
4	644/08	+	3	3	6	+	3	2	5	+	4	3	12
5	763/08	+	3	3	6	+	1	1	2	+	2	2	4
6	857/08	+	3	3	6	+	2	2	4	+	3	3	9
7	926/08	+	2	3	5	+	2	3	5	+	3	3	9
8	1023/08	+	2	3	5	+	2	3	5	+	3	3	9
9	1369/08	+	2	2	4	+	1	1	2	-	1	1	1
10	1601/09	+	4	3	7	+	3	3	6	+	4	4	16
11	1604/09	+	4	3	7	+	1	2	3	+	2	2	4
12	1608/08	+	4	3	7	+	4	3	7	+	4	4	16
13	1943/08	+	3	2	5	+	1	2	3	+	2	3	6
14	66/09	+	4	3	7	+	3	3	6	+	3	4	12
15	2328/08	+	4	3	7	+	4	3	7	+	3	3	9
16	2348/08	+	4	3	7	+	1	3	4	+	2	3	6
17	2379/08	+	3	3	6	+	3	3	6	+	4	4	16
18	2525/08	+	3	3	6	+	2	3	5	-	1	1	1
19	2581/08	+	3	3	6	+	3	3	6	+	2	2	4
20	2717/08	+	2	1	3	+	2	2	4	+	3	3	9
21	2718/08	+	3	3	6	+	3	3	6	+	3	3	9
22	2765/08	+	3	2	5	+	2	2	4	+	3	4	12
23	2798/08	+	2	1	3	+	2	2	4	+	3	4	12
24	2817/08	+	2	3	5	+	2	3	5	+	3	4	12
25	2827/08	+	3	1	4	+	2	2	4	+	3	3	9

Exp- Expression; Ext- Extent; Int-Intensity; TS- Total Score.

Table X shows the distribution (extent and intensity) of the molecular markers in our study population. The intensity and extent of staining was quantified systematically as mentioned before. A total score was derived from the intensity and extent of staining. For p53 and cyclin D1 the total score was calculated as the sum of the individual scores obtained for the extent and intensity of staining. Whereas for CD44 it is the product of the individual scores obtained for the extent and intensity of staining. All the cases exhibited positive staining for p53 and Cyclin D1 as compared to CD44 that was negative for immunostaining in 4 cases. Although majority of the cases were positive for immunostaining the extent and intensity of the staining varied among the cases.

Table XI: Distribution of immunohistochemical scoring in Oral SCC

Molecular Marker		P53				Cyclin D1				CD44				Total Cases
Total Score		0	1-3	4-5	6-7	0	1-3	4-5	6-7	1-4	5-8	9-12	13-16	
In situ		0	0	0	2	0	0	1	1	0	1	0	1	2
Invasive oral SCC	WD	0	1	2	4	0	2	4	1	4	0	3	0	7
	MD	0	1	3	6	0	1	5	4	3	1	5	1	10
	PD	0	0	0	2	0	1	0	1	1	0	0	1	2
Verrucous carcinoma		0	0	3	1	0	0	4	0	0	0	4	0	4
Total		0	2	8	15	0	4	14	7	8	2	12	3	25

WD: Well Differentiated; MD: Moderately Differentiated; PD-Poorly Differentiated

Table XI shows the distribution of the total score for the molecular markers p53, cyclin D1 and CD44. Total scores obtained from the immunohistochemical study for the 3 markers were analyzed to find the frequency of distribution of the scores among the different grades of oral SCC and in verrucous carcinoma.

p53 immunostaining revealed that the total score was predominantly in the higher side in insitu and invasive SCC. But, three fourths of verrucous carcinoma had their total scores in the mid-range (4 to 5). Cyclin D1 immunostaining revealed that the total score was predominantly in the mid-range (total score-4 to 5) including all the cases of verrucous carcinoma. CD44 immunostaining revealed that the total score was predominantly in the range of 9 to 12.

Table XII: Relationship between over expression of molecular markers and Histological Grade in invasive oral cancer

Histological grade	N	P53 over expression (Total Score ≥ 4)		CyclinD1 over expression (Total Score ≥ 4)		CD44 Strong positivity (Total Score > 4)	
		Absent	Present	Absent	Present	Absent	Present
Well differentiated	7	1	6	2	5	4	3
Moderately differentiated	10	1	9	1	9	3	7
Poorly differentiated	2	0	2	1	1	1	1
Total cases	19	2	17	4	15	8	11

Table XII shows the relationship between over expression of molecular markers and histological grade in invasive oral cancer. For the complexity of analysis of the different ranges in the total scores of the staining of the 3 molecular markers a simplified method was followed as mentioned before in the scoring system. A cut off value for total score of 3 for p53 and Cyclin D1 and 4 for CD44, above which the molecular markers were defined as overexpressed. 89%, 79% and 58% of the cases of invasive SCC were found to overexpress p53, Cyclin D1 and CD44 respectively.

Table XIII: Overexpression of molecular markers in Insitu and Invasive SCC vs Verrucous Carcinoma

Overexpression of Molecular Markers	Insitu and Invasive SCC	Verrucous Carcinoma
P53	19/21	4/4
Cyclin D1	17/21	4/4
CD44	13/21	4/4

Table XIII shows a comparison of molecular markers over expression in SCC and its variant verrucous carcinoma.

We wanted to analyze if there is any statistically significant relationship between the tumor grade and the degree of expression of the molecular markers. To simplify the analsysis we studied the correlation of the grade of the tumor with the presence or absence of over expression of the molecular markers.

Table XIV: Correlation of Histological Grade with degree of expression of Molecular Markers in Invasive Oral SCC

		Total P53 Score	Total Cyclin D1 Score	Total CD44 Score
Grade	**Correlation**	0.379	0.232	0.192
	P value	0.110	0.339	0.430
	Number of Cases	19	19	19

Table XIV shows the statistical Pearsons correlation of histological grade with the presence or absence of over expression of molecular markers.

The analysis revealed insignificant statistical correlation between the histological grades and over expression of molecular markers. Within the molecular markers, over expression of p53 showed slightly higher correlation (p=0.110) than that of cyclin D1 and CD44 although without any statistical significance. Comparing Cyclin D1 and CD44, Cyclin D1 (0.339) showed better correlation than CD44 (0.430) again without any statistical significance.

Table XV: Expression of molecular markers in patients with cervical node metastasis

HPE No	P53			Cyclin D1			CD 44		
	Ext	Int	TS	Ext	Int	TS	Ext	Int	TS
644/08	3	3	6	3	2	5	4	3	12
2328/08	4	3	7	4	3	7	3	3	9
2379/08	3	3	6	3	3	6	4	4	16
2525/08	3	3	6	2	3	5	1	1	1
119/09	3	2	5	3	2	5	1	1	1
120/09	3	3	6	1	3	4	2	2	4
2581/08	3	3	6	3	3	6	2	2	4

EXP- Expression; EXT-Extent; INT-Intensity; TS- Total Score

Table XV gives an idea about the expression of molecular markers in case of invasive SCC with regional cervical node metastasis. Overexpression of p53 and Cyclin D1 was observed in all the 7 cases with nodal metastasis whereas strong positivity for CD44 was observed only in 3 cases.

Table XVI: Correlation of Biomarker Expression and cervical node metastasis in Invasive Oral SCC

Biomarker	Total score	No of cases	Nodal Metastasis		Chi Sq value	P value
			Present	Absent		
P53	<4	2	0	2	0.135	0.714
	≥4	17	7	10		
Cyclin D1	<4	4	0	4	1.290	0.256
	≥4	15	7	8		
CD44	≤4	8	4	4	0.283	0.594
	>4	11	3	8		

Table XVI shows the statistical analysis done to look for any significant relationship between degree of expression of molecular markers and the status of the nodal involvement. Chi-square test was used to evaluate the statistical significance. None of the 3 molecular markers expressed showed any statistically significant relationship with the nodal metastasis.

Table XVII: Relationship between p53, cyclin D1 and CD44 expression in oral cancer

Molecular markers	n	P53			Cyclin D1		
CyclinD1		<4	≥4	p Value	<4	≥4	P Value
<4	4	0	4	0.868			
≥4	21	2	19				
CD44							
≤4	8	0	8	0.872	3	5	0.154
>4	17	2	15		1	16	

Table XVII shows the statistical analysis done to study the relationship between expression of all the 3 molecular markers in oral SCC and its variant verrucous carcinoma. Results were found to be statistically insignicant.

Table XVIII: Expression of molecular markers in verrucous oral cancer

HPE NO	SITE	P53				CYCLIN D1				CD44			
		EXP	EXT	INT	TS	EXP	EXT	INT	TS	EXP	EXT	INT	TS
1023/08	4	P	2	3	5	P	2	3	5	P	3	3	9
2817/08	1	P	2	3	5	P	2	3	5	P	3	4	12
857/08	4	P	3	3	6	P	2	2	4	P	3	3	9
926/08	1	P	2	3	5	P	2	3	5	P	3	3	9

SITE 4: Buccal Mucosa, 1: Lip

Table XVIII shows the expression and scoring of immunostaining of the various molecular markers in cases of verrucous carcinoma. All the 3 molecular markers were over expressed in all the 4 cases. The staining for the molecular markers was predominantly observable in the pushing margin of the tumor in most of the cases reflecting the maximum proliferative activity of this tumor in these areas. The staining tends to diminish in the superficial areas that could represent the more differentiated part of the tumor where the cells have stopped proliferating.

DISCUSSION

The majority of oral cancers are SCC, and numerous studies have been done on them with a prime objective of understanding the biology, diagnosis, prognosis, and management of this entity.[140] Verrucous carcinoma (VC) is a low-grade variant of squamous carcinoma that shows distinct clinical and histological features and whose molecular alterations have not been extensively studied. The multistage process of carcinogenesis involves the progressive acquisition of mutations and epigenetic abnormalities in the expression of multiple genes that have highly diverse functions. An important group among these genes is involved in cell cycle control.[141] So, we have studied the role of cell cycle regulators p53, cyclin D1 and the cell adhesion molecule, CD44 in tumor differentiation and regional node metastasis of oral SCC.

AGE OF OCCURRENCE:

The mean age of the patients included in our study was 57.08 years (Range 40-80 years). This is more or less similar to the observation made by Xu et al in which the mean age of the study group was 60 years (Range 34-86).[142] The mean age of presentation in another study done by Lam et al on oral SCC was 64 years (Range 37-85 years).[143]

TOBACCO AND ALCOHOL USAGE:

In the study done by Xu et al on human oral carcinomas they found that all patients had a history of considerable current or previous use of tobacco use and 72% of the patients had history of alcohol abuse. [142]

In a study done by Maahs et al on 45 patients with oral SCC, 80% and 56% of them had history of tobacco and alcohol use respectively.[144]

Similarly, in our study some form of tobacco use was found in 92% of patients and alcohol use was found in 40% of patients. A slightly lesser percentage of alcohol use could be related to the higher number of female patients (M: F= 1.08:1) included in our study who are less likely to consume alcohol compared to the western population.

SITE DISTRIBUTION:

In a study done by Ozer et al on oral SCC the frequent tumor sites were tongue (40%), lips (25%) and gingiva (25%). In another study done by Lam et al on oral SCC the commonest sites of tumor were floor of the mouth and buccal mucosa.[143]

The commonest tumor sites in our study group were tongue and buccal mucosa each contributing to about a quarter of patients and the least common site was floor of the mouth (4%). Rest of the sites showed almost equal distribution (around 10%).

Vicente et al studied the significance of p53 expression in oral SCC without neck node metastases in 91 patients with oral SCC. In their study

well, moderately and poorly differentiated SCC contributed to 62%, 25% and 4% of the cases respectively.[145]

In a study done by Sano et al on 37 patients with oral SCC, well and moderately differentiated carcinoma and verrucous carcinoma contributed to 73%, 21% and 6% of cases respectively.[146]

In our study 75% of the tumors were invasive SCC and among the remaining quarter of the specimens verrucous carcinoma and in situ carcinoma contributed to 16 and 8%. Among the invasive SCC well, moderate and poorly differentiated formed 37%, 53% and 10% respectively.

NECK NODE METASTASIS:

Maahs et al in their study on 45 patients with SCC of mouth reported metastatic neck node involvement in 51% of their cases. [144]

In another study done by Vicente et al on 91 cases of oral SCC nodal involvement was found in 37% of cases.[145]

Similarly, in our study, metastatic regional lymph node involvement was present in 37% of patients with invasive oral SCC.

EXPRESSION OF MOLECULAR MARKERS IN THE ADJACENT NON-TUMORAL EPITHELIUM AND CARCINOMA IN SITU:

p53:

Gonzalez-Moles et al [147] found that all normal epithelia adjacent to the tumor were p53-negative. p53 expression was detected in 25% of cases of adjacent hyperplastic epithelia and in 43% of cases with dysplasia adjacent to

the tumor. They concluded that the number of cases that expressed p53 increased progressively with the severity of the epithelial disorder until the maximum percentage of positivity in tumor tissue was reached.

Kerdpon et al in their study about the expression of p53 in oral mucosal hyperplasia, dysplasia and SCC observed a difference in staining pattern between the two groups, as p53-positive cells in lesions with benign hyperplasia generally were located in the basal and suprabasal layers, whereas dysplasias showed a varied staining, from confinement of p53-positive cells to the basal and parabasal layers in mild dysplasia to widespread staining in severe dysplasias.[148]

In our study, the normal or hyperplastic epithelium adjacent to the tumor showed p53 expression predominantly in the basal layer in 60% of cases and in both the basal and suprabasal layers in rest of the cases. The intensity of staining was variable. In sections showing dysplastic changes in the adjacent epithelium there was a strong nuclear expression within the entire dysplastic area. Both the cases of carcinoma in situ studied showed full thickness strong nuclear expression for p53.

Cyclin D1:

Kuo et al found that positive cyclin D1 stain could be found in the normal appearing epithelium immediately adjacent to the SCCs in 54 of 73 (74%) cyclin D1-positive cases and concluded that over expression of cyclin D1 may be an early event in oral carcinogenesis.[149]

Kotelnikov et al found that cyclin D1 positive cells are located mainly in suprabasal layers of oral epithelium in both truly normal and tumor-associated mucosa and also that the frequency of cyclin D1 positive cells in the basal layer is very low. [150] This was comparable to the finding in our study. This might be explained by the fact that only cells in suprabasal layers are proliferatively active in human oral epithelium from SCC patients.

In our study, cyclin D1 expression in the adjacent normal or hyperplastic epithelium was predominantly present in the suprabasal layer in 65% of the cases and was present in both the basal and suprabasal layers in rest of the cases. There was increased expression of cyclin D1 in all the 3 cases with mild to moderate dysplasia in the adjacent epithelium. The increased expression of cyclin D1 was confined to the areas showing dysplasia. Of the2 cases of carcinoma in situ, one showed full thickness strong nuclear expression for cyclin D1 whereas in the other case the strong nuclear expression was restricted to the suprabasal layer. The difference in expression of cyclin D1 in these 2 cases could not be explained.

CD44:

Roye et al studied the CD44 in dysplastic epithelium and SCC of esophagus and found that in normal (uninvolved) epithelium, immunostaining was strongest in the basal-cell layer and bottom third and weak or absent in the superficial layers. There was a significant increase in the intensity of immunostaining throughout all levels of the dysplastic epithelium, and the extent of this increase correlated directly with the severity of the dysplasia.[151]

Bankfalvi et al, in their study on gains and losses of adhesion molecules, found that there is constitutive expression of CD44 in the basal layer and lower part of the stratum spinosum in non-neoplastic oral epithelium. And they also found that there was increased expression of adhesion molecules with increase in dysplasia.[152]

In our study, the CD44 staining pattern was predominantly confined both to the basal and suprabasal layers in the adjacent non-tumoral epithelium that was normal or hyperplastic. The dysplastic adjacent epithelium showed increased expression of CD44. Among the 2 cases of carcinoma in situ studied, one of them showed full thickness strong membranous expression for CD44 whereas in the other the staining was restricted to the basal layers.

EXPRESSION OF MOLECULAR MARKERS IN ORAL SCC:

Results from the study by Xu et al suggest that p53 mutations and cyclin D1 overexpression are common genetic events in oral cancer with 65% and 39% positivity for p53 and cyclin D1 respectively.[142]

p53 and cyclin D1 expression was found in 46% and 39% respectively of the 140 patients with oral SCC studied by Shiraki et al.[153]

In our study, p53, cyclin D1 and CD44 expression was present in 89%, 79% and 58% respectively of the 19 cases with invasive SCC.

CORRELATION OF MOLECULAR MARKER EXPRESSION WITH HISTOLOGICAL GRADING AND NODAL METASTASIS

p53:

Nishioka et al [154] on a study in patients with oral SCC found a relationship between p53 expression and tumor grade, but not with the tumor stage. Vicente et al, in a study done on oral SCC, found a correlation of p53 protein immunoexpression with histologic grade, but not with other clinical variables.[145]

In contrast to the above finding, Yan et al in a study on carcinoma of buccal mucosa and tongue reported no correlation between p53 expression and tumor grade, disease stage, node status, or early local recurrence.[155] Similarly Lam et al in their study found no significant correlation between p53 expression and grade of oral SCC.[143]

Unal et al, in their study on p53 expression and histopathological parameters in SCC of tongue, found that p53 positivity statistically correlated with nodal metastasis.[156]

Shiraki et al, in their study on oral cancer, found no statistical significance between p53 positivity and nodal metastasis.[153]

In our study although over expression of p53 showed slightly higher correlation with the tumor grade than that of the cyclin D1 and CD44, it did not reach statistical significance. Similarly, there was no statisitically significant association between p53 over expression and nodal metastasis.

Cyclin D1:

Angadi et al in their study regarding the cyclin D1 expression in various grades of oral SCCs reported a proportional increase in the percentage of positivity with increase in the cytological grade.[157] This was in accordance with 2 other studies of oral SCC by Lam et al [143] and Miyamoto et al.[158]

In a study by Wu et al.,[159] cyclin D1 positivity was seen in 100% of well-differentiated SCC, 67% of moderately differentiated SCC, and 50% of poorly differentiated SCC, taking into consideration the number of positive cells. This study suggested a positive correlation of cyclin D1 expression with degree of differentiation, which is in contrast with our study where the cyclin D1 expression was correlating with the lack of differentiation of the tumor and increased with poor histological grade, that is, from well-differentiated SCCs to poorly differentiated SCCs.

A similar study by Castle et al[160] found no correlation with the degree of differentiation, as none of the poorly differentiated SCCs exhibited intense staining.

In the literature, the association between cyclin D1 expression and the presence of neck node metastasis is ambiguous. Maahs et al studied expression of cyclin D1 by using immunohistochemical methods in 45 patients with epidermoid carcinoma of the mouth and concluded that there is no association between expression cyclin D1 and neck node metastasis.[144] The results of the present investigation agree with those presented by Han et al,[161] Michalides et al[162, 163] and Bellacosa et al.[164]

However, there are papers from Frachiolla, [165] Itami,[166] Masuda,[167] Tapia et al, Capaccio et al,[168] and Mineta et al[169] showing that expression of D1 cyclin is indeed associated with the presence of neck node metastasis. Results are contradictory and the reasons are not clear.

Rodolico et al in their study 122 cases of lower lip SCC observed a 67% cyclin D1 protein positivity; moreover multivariate analysis identified a significant independent predictive value for lymph node metastases occurrence.[170]

Bova et al in their study on 148 tongue cancer patients showed that overexpression of cyclin D1 occurred in 68% of tumors and was associated with increased lymph node stage and increased tumor grade.[171]

In our study, there was no statistically significant relationship between cyclin D1 over expression with nodal metastasis or histological grade despite cyclin D1 over expression in 79% of cases of invasive oral SCC.

CD44:

Bankfalvi et al found that loss of CD44 and other adhesion molecules may be necessary to develop a definitively malignant phenotype with the ability to invade adjacent tissues. They concluded that some of the qualitative changes of adhesion molecule phenotype significantly signal poor outcome and/or forecast metastasis. [152] This fact was substantiated by the study on 36 patients with SCC of tongue by Gonzalez-Moles et al that suggested a critical role for these invasion-related molecular processes in the prognosis of tongue

cancer patients. They stated that loss of CD44 and other adhesion molecules may be necessary to develop a definitively malignant phenotype with the ability to invade adjacent tissues.[172]

Kunishi et al in their study concluded highly differentiated carcinomas displayed more intense CD44v6 reactivity than less differentiated ones without statistical significance. Their study also showed that oral SCC with low expression of CD44v6 more often had regional metastatic potential and may be related to lymph node metastasis. They all concluded that low expression of CD44v6 in oral SCC tissues may be an indicator of high metastatic potential and may also be related to lymph node metastasis.[173]

Roye et al studied the CD44 expression in dysplastic epithelium and squamous cell carcinoma of the esophagus and observed that there was a decreased expression of CD44 in poorly differentiated esophageal SCC that may indicate an association of loss of CD44 expression with tumor progression.[151]

Ozer et al in their study of 20 cases of oral SCC demonstrated that CD44 expression by tumor cells in oral cavity carcinomas statistically correlated with nodal metastasis. (ÖZER E., KUYUCUO–LU F. Correlation of CD44 expression with prognostic factors in oral squamous carcinoma. Eastern Journal of Medicine 4 (2): 61-64, 1999.)

In the present study we did not find any statistically significant correlation of CD44 positivity with either histological grade or nodal metastasis.

VERRUCOUS CARCINOMA:

Verrucous carcinoma of the oral cavity should be considered as a clinicopathologic entity, distinct from the more common SCC because of its unique biologic behavior. This tumor shows much slower growth with a limited propensity to metastasize and hence offers a better prognosis than SCC. Significant clinical and biologic differences are found between invasive SCC and verrucous carcinoma, which we attempted to evaluate using p53, cyclin D1 and CD44.

In a study done by Angadi et al on Cyclin D1 expression in oral SCC and verrucous carcinoma, they found that 63% of the verrucous carcinoma was positive for cyclin D1. They also observed that the predominant intensity observed was mild.[157]

In another study done by Gimenez-Conti et al in oral verrucous carcinoma 50% of them were positive for p53 staining and 61% of them expressed cyclin D1.[174]

Ogawa et al recorded a difference in CD44v9 expression between verrucous carcinoma and well differentiated oral SCC of the tongue which was more frequently found in verrucous carcinoma. They concluded that this difference may be responsible for the variation in their metastatic capacity between these tumor types.[175]

In our study, the 4 cases of verrucous carcinoma showed over expression of all the 3 molecular markers and the predominant intensity was moderate.

CONCLUSION

1. The mean age of incidence of oral SCC in our study was 57 years with almost equal sex distribution.
2. Ninety-two percent of the patients were tobacco users and 40% used alcohol.
3. The most frequent sites of tumor within the oral cavity were tongue and buccal mucosa.
4. Most (75%) of the cases were invasive SCC. Verrucous and insitu carcinomas contributed to 8% and 16% of cases respectively.
5. Metastasis to the regional lymph node was found in 37% of cases of invasive SCC.
6. Dysregulation of cell cycle regulatory proteins p53 and cyclin D1 and the cell adhesion molecule CD44 appeared to be common events taking place in oral SCC.
7. The increased expression of these molecular markers in the adjacent non-tumoral epithelium and cases of carcinoma insitu suggested that the dysregulation of these proteins are early events in oral carcinogenesis.
8. However, there was no statistically significant relationship between the expression of these molecular markers and histological grading.
9. Also, in our study there was no statistical significance between expression of molecular markers and regional lymph node metastasis.
10. The abnormal over expression of these molecular markers were found to be common even in verrucous carcinoma, a low grade variant of well differentiated oral SCC.

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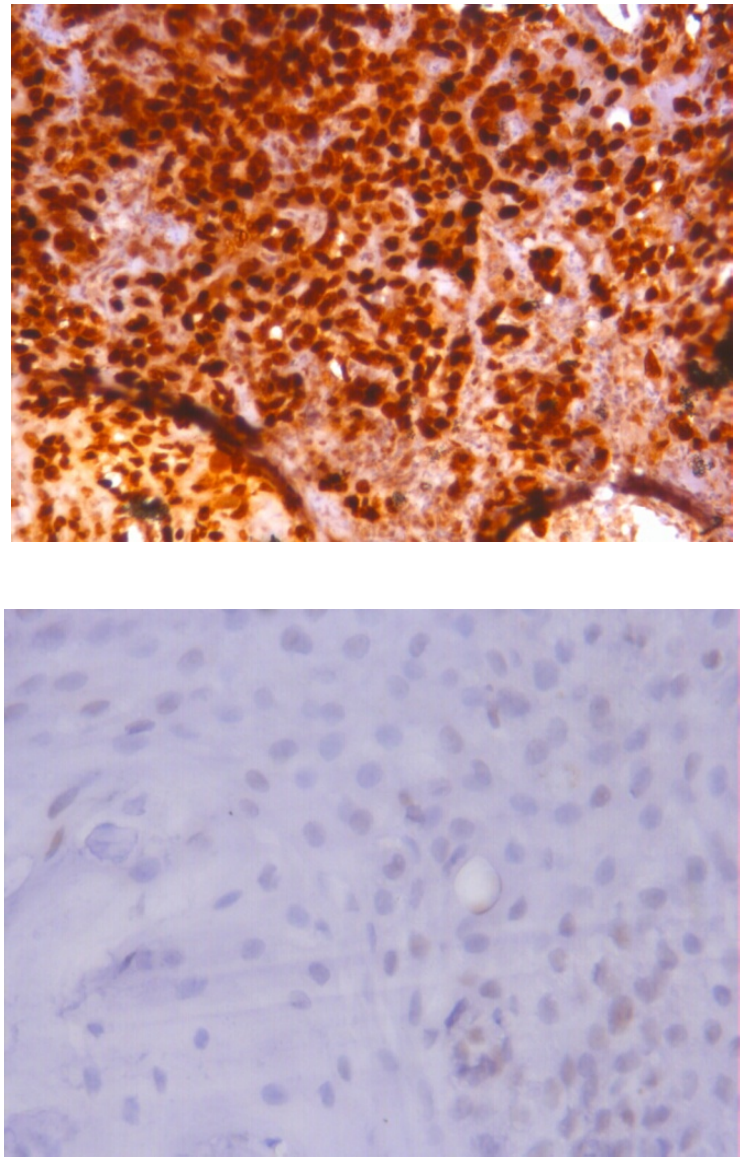

Figure 9: Photomicrograph of the p53 immunohistochemical staininig in oral SCC (Counter-stain used: Hematoxylin). The upper panel (Magnification: 200X) shows a brown colored positive staining of the malignant cells for p53. A nuclear pattern of staining is observed. In contast, the bottom panel (Magnification: 400x) shows a negative immunostaining for p53. Few scattered tumor cell nuclei exhibit very faint positivity for p53 with no immunoreactivity in most of the cells.

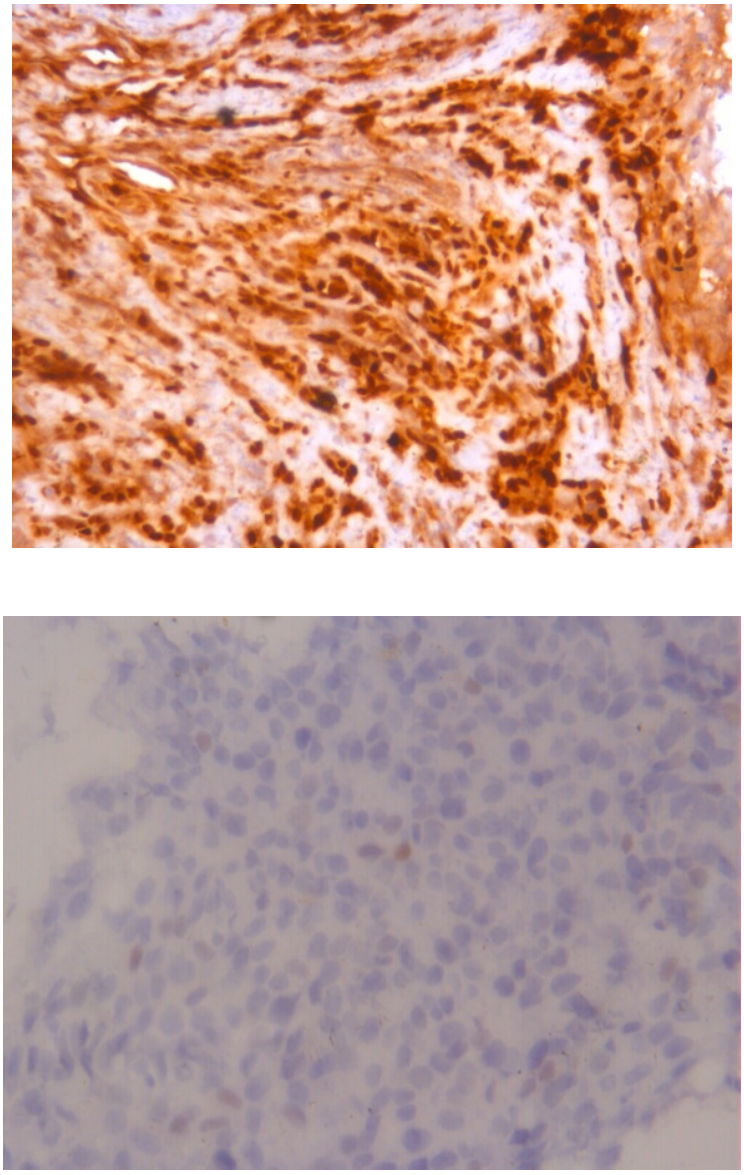

Figure 10: Photomicrograph of the Cyclin D1 immunohistochemical staininig in oral SCC (Counter-stain used: Hematoxylin). The upper panel (Magnification: 200x) shows brown colored positive staining of the malignant cells for Cyclin D1. A nuclear pattern of staining is observed. In contrast, the bottom panel (Magnification: 400x) shows a negative immunostaining for Cyclin D1. Few scattered tumor cell nuclei exhibit very faint positivity for Cyclin D1 with no immunoreactivity in most of the cells.

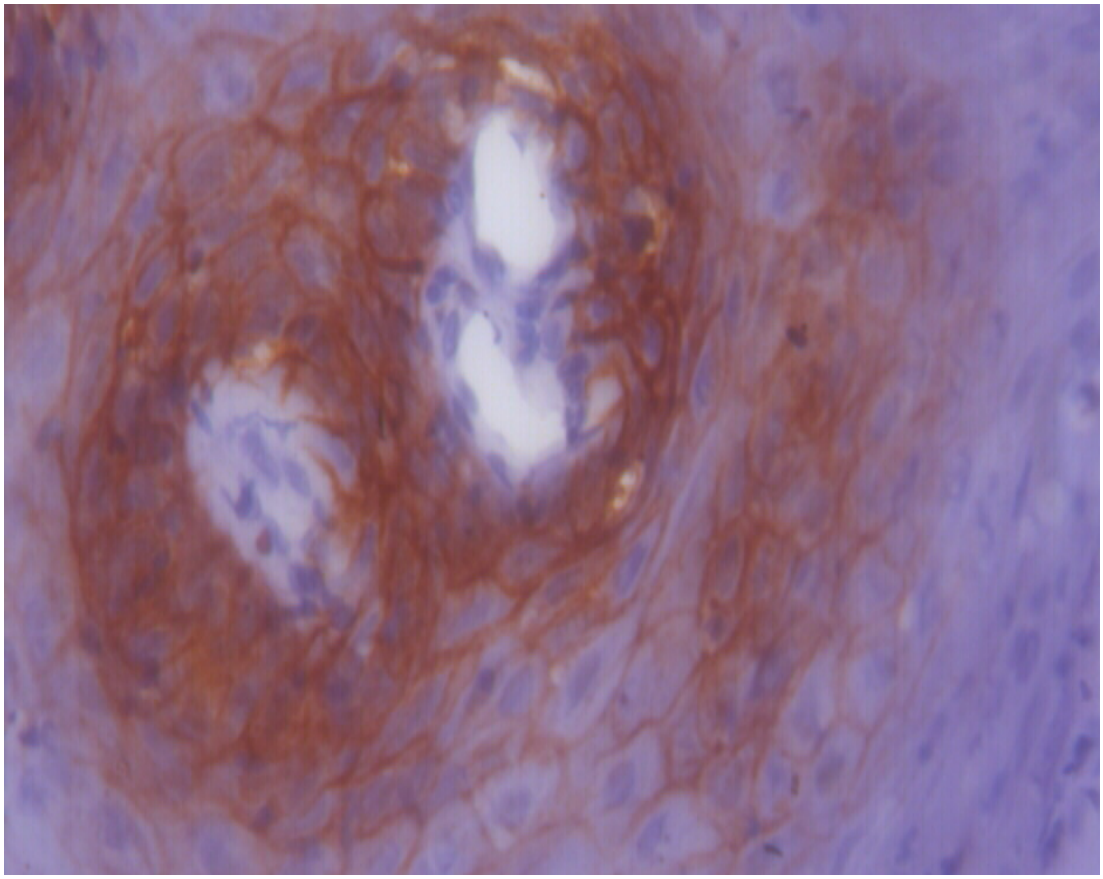

Figure 11: Photomicrograph of the CD44 immunohistochemical staininig in oral SCC (Counter-stain used-Hematoxylin; Magnification: 400x). The picture shows brown colored positive staining for CD44. It is restricted to the cell membranes of the tumor cells.

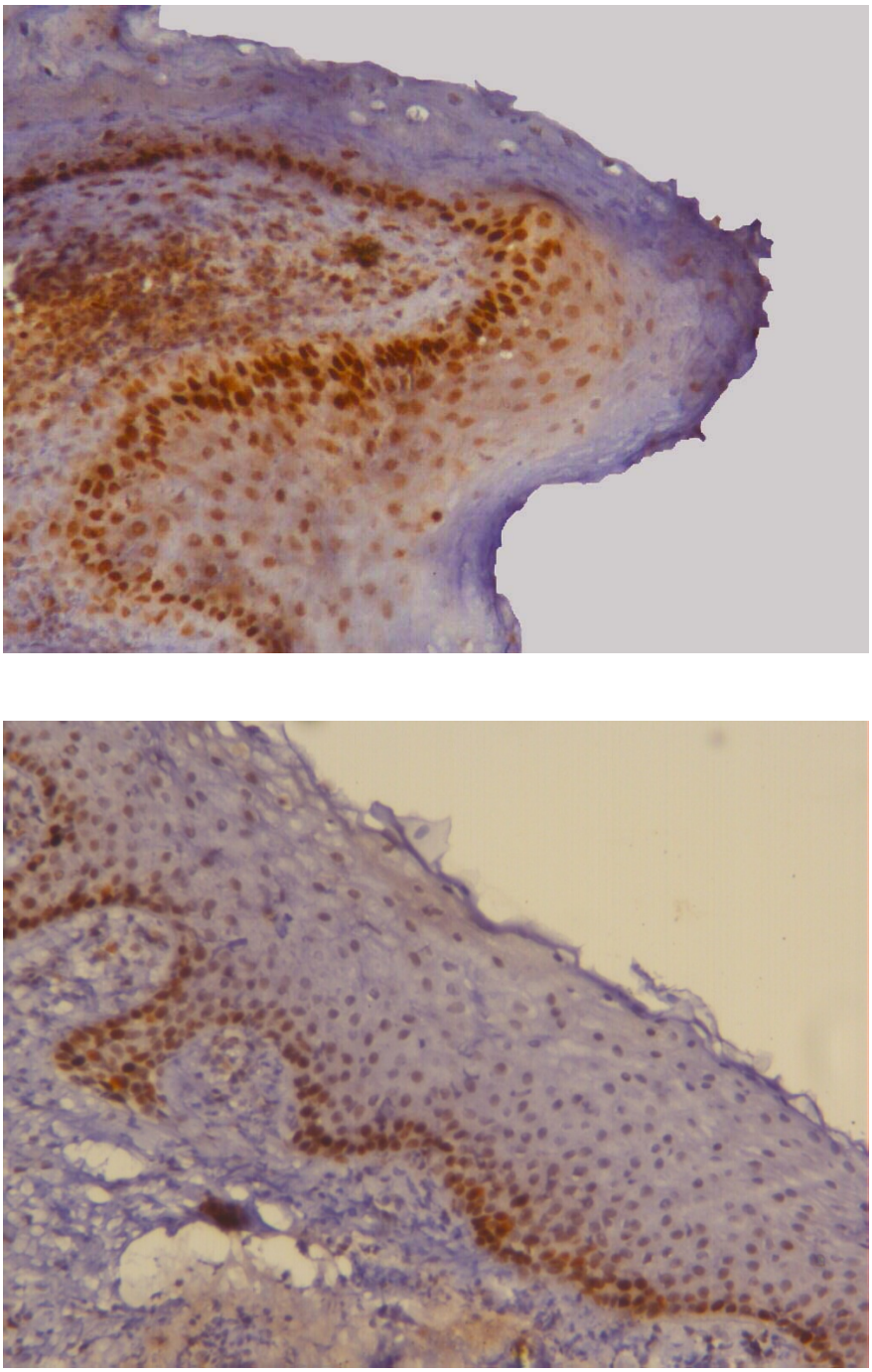

Figure 12: Photomicrograph of p53 immunohistochemical staining in the adjacent non-tumoral epithelium (Magnification-200x) in 2 different cases. Brown colored nuclear pattern of staining is observed in both the basal and suprabasal layers in both the cases.

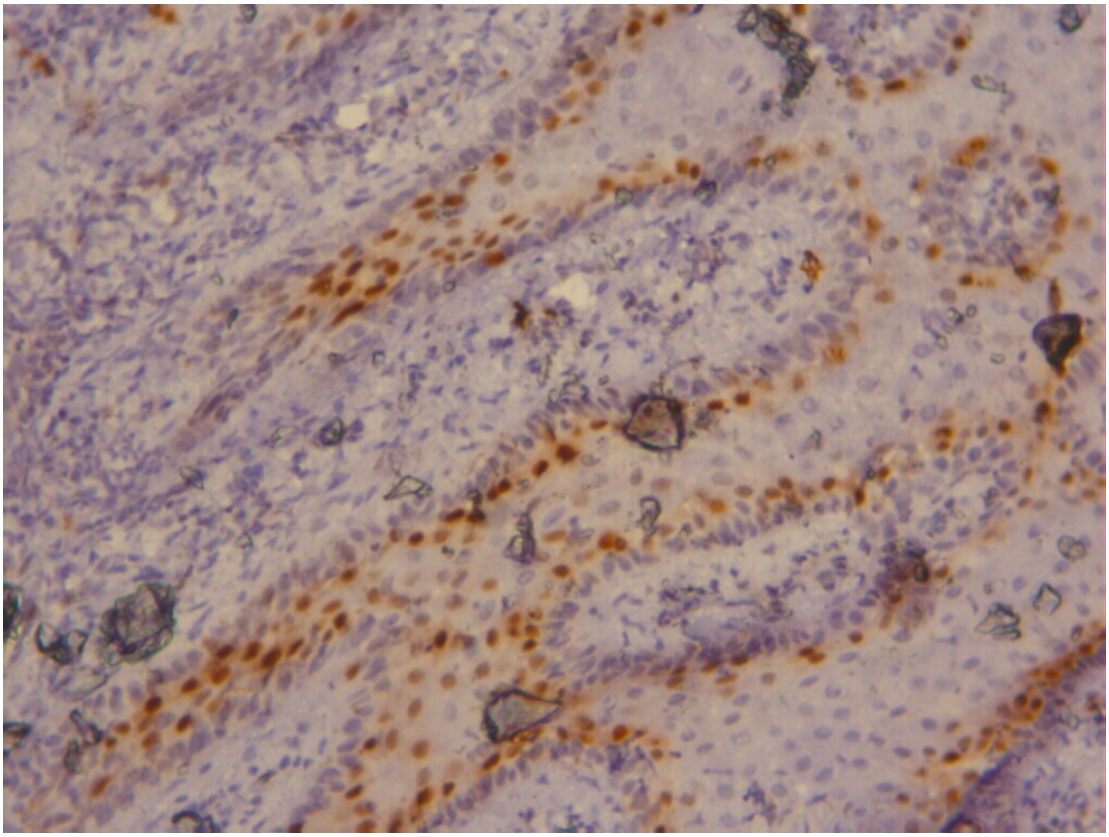

Figure 13: Photomicrograph of Cyclin D1 immunohistochemical staining in the adjacent non-tumoral epithelium (Magnification-200x). Brown colored nuclear pattern of staining is observed predominantly in the suprabasal layers.

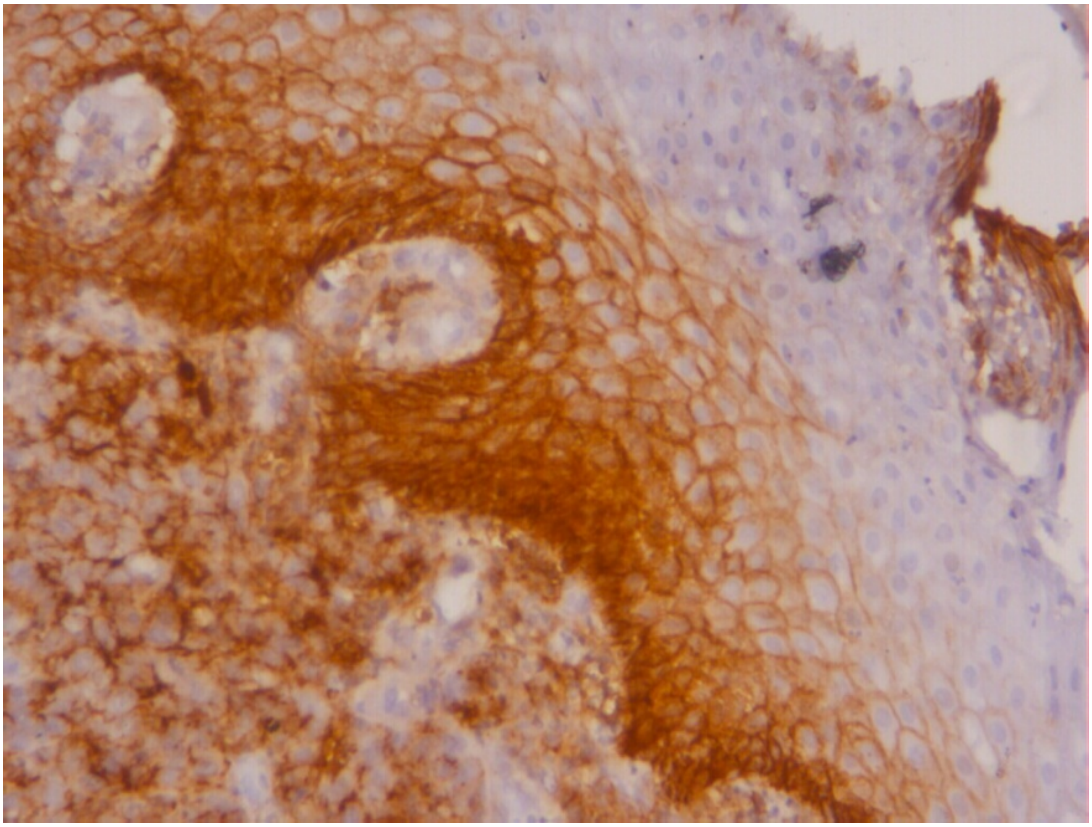

Figure 14: Photomicrograph of CD44 immunohistochemical staining in the adjacent non-tumoral epithelium (Magnification-200x). Brown colored membranous pattern of staining is observed in the basal and suprabasal layers.

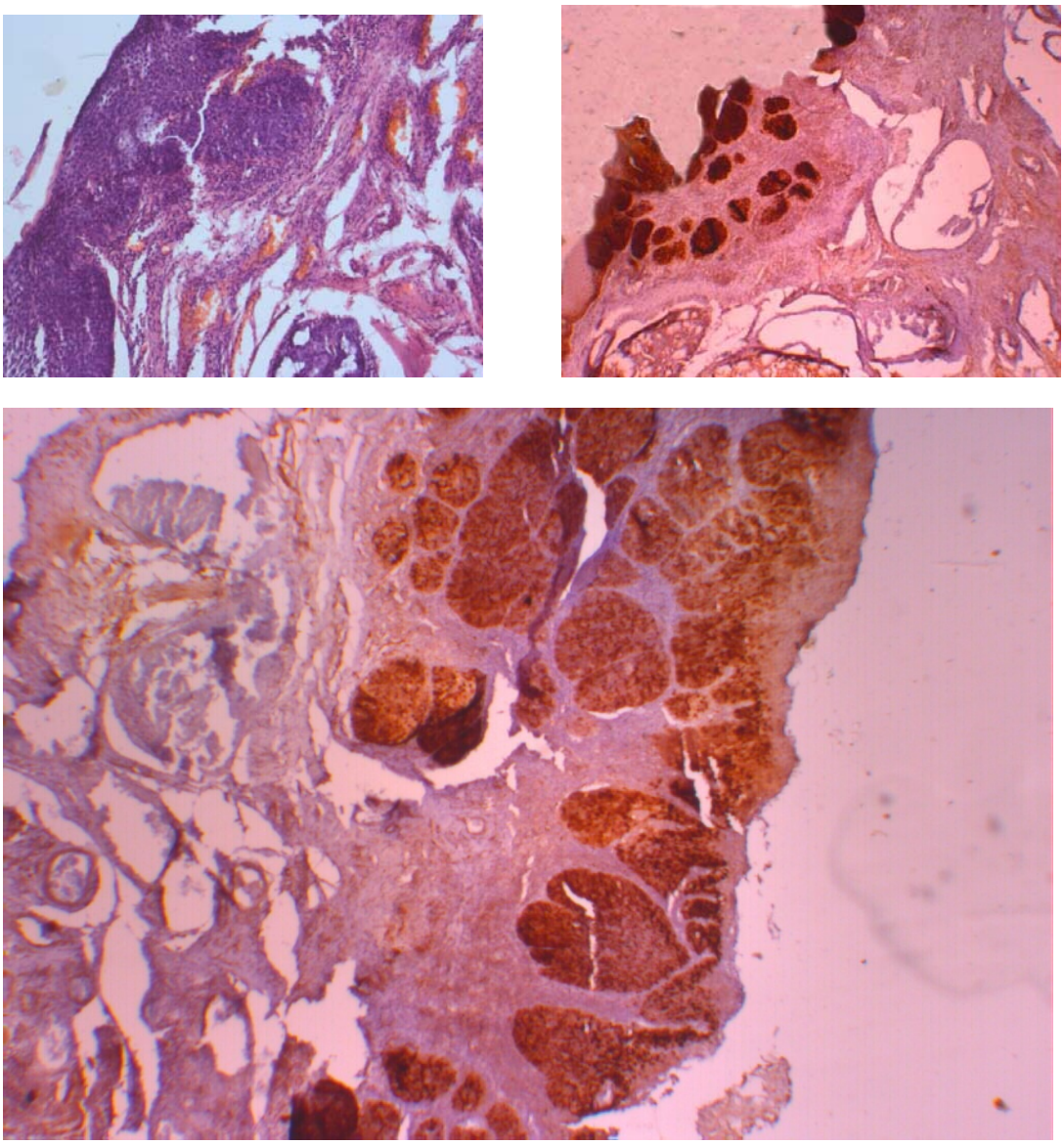

Figure 15: Photomicrograph of Squamous cell carcinoma insitu. Upper left panel: H&E section showing full thickness dysplasia**.** Upper right panel: Immunostain for p53 - entire thickness of the epithelium shows strong positivity. Bottom panel: Immunostain for Cyclin D1- entire thickness of the epithelium shows strong positivity.

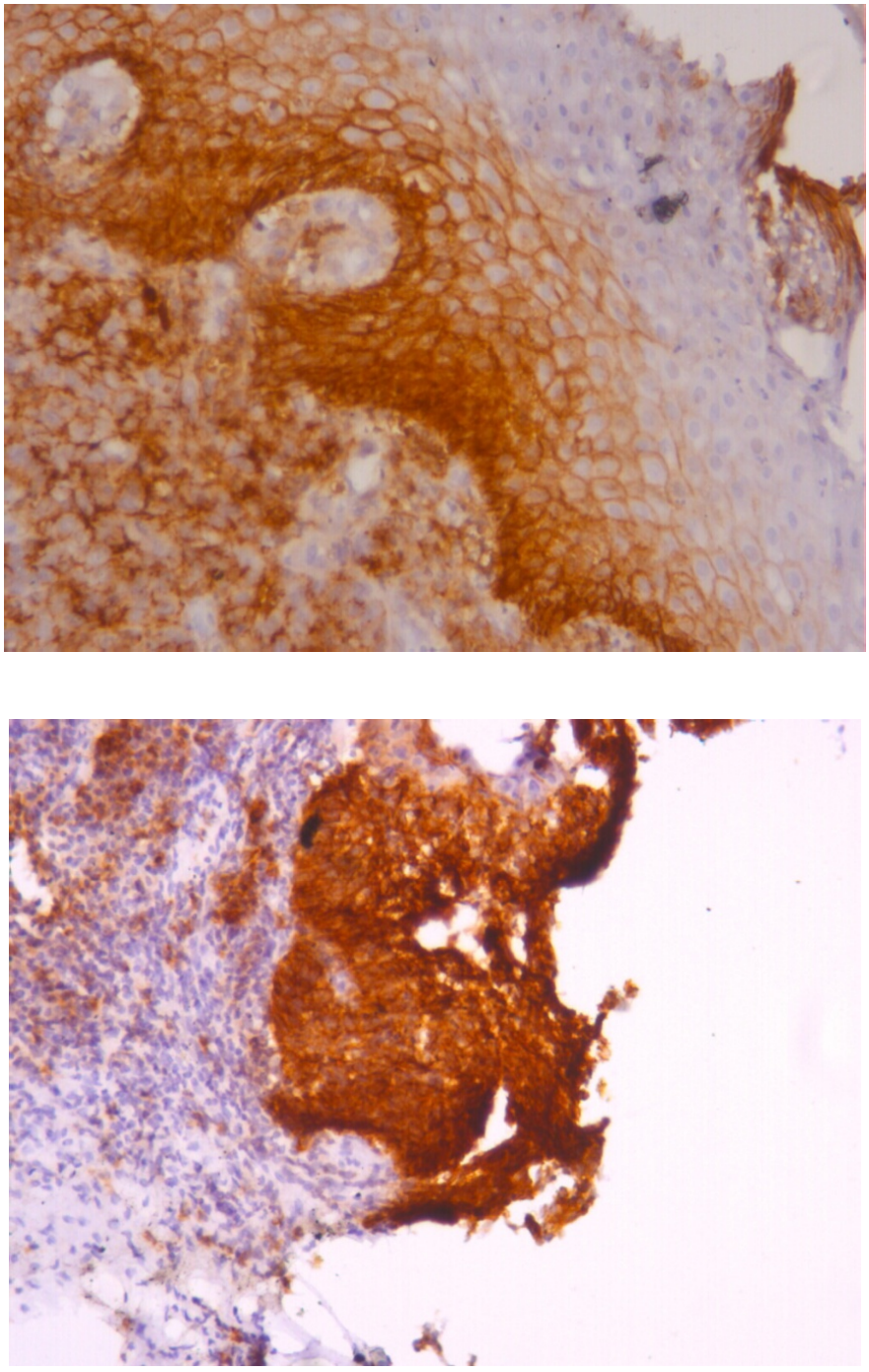

Figure 16: Photomicrograph of CD44 immunostaining. Upper panel shows basal and suprabasal expression of CD44 in adjacent Non-Tumoral epithelium. In contrast lower panel shows intense full-thickness CD44 positivity in a case of squamous cell carcinoma insitu.

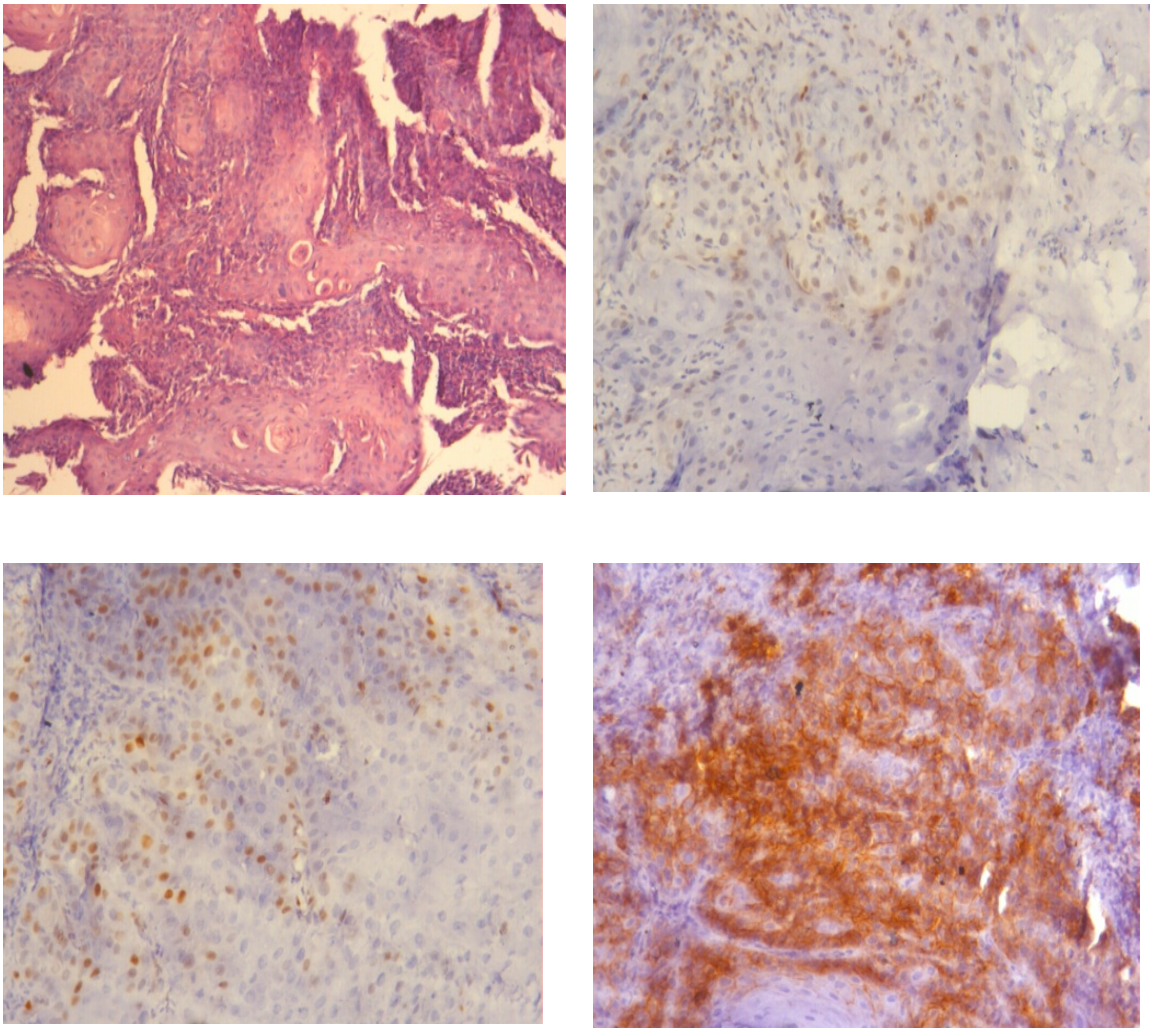

Figure 17: Photomicrograph of well differentiated OSCC arising from the tongue. Upper left panel: H&E section showing well keratinized tumor cells and few epithelial pearls. Upper right panel: Most of the tumor cells are negative for p53 immunostaining except a few. Bottom left panel: Nearly 1/3rd of the tumor cells showing moderate cyclin D1 positivity. Bottom right panel: Diffuse positivity for CD44 immunostaining.

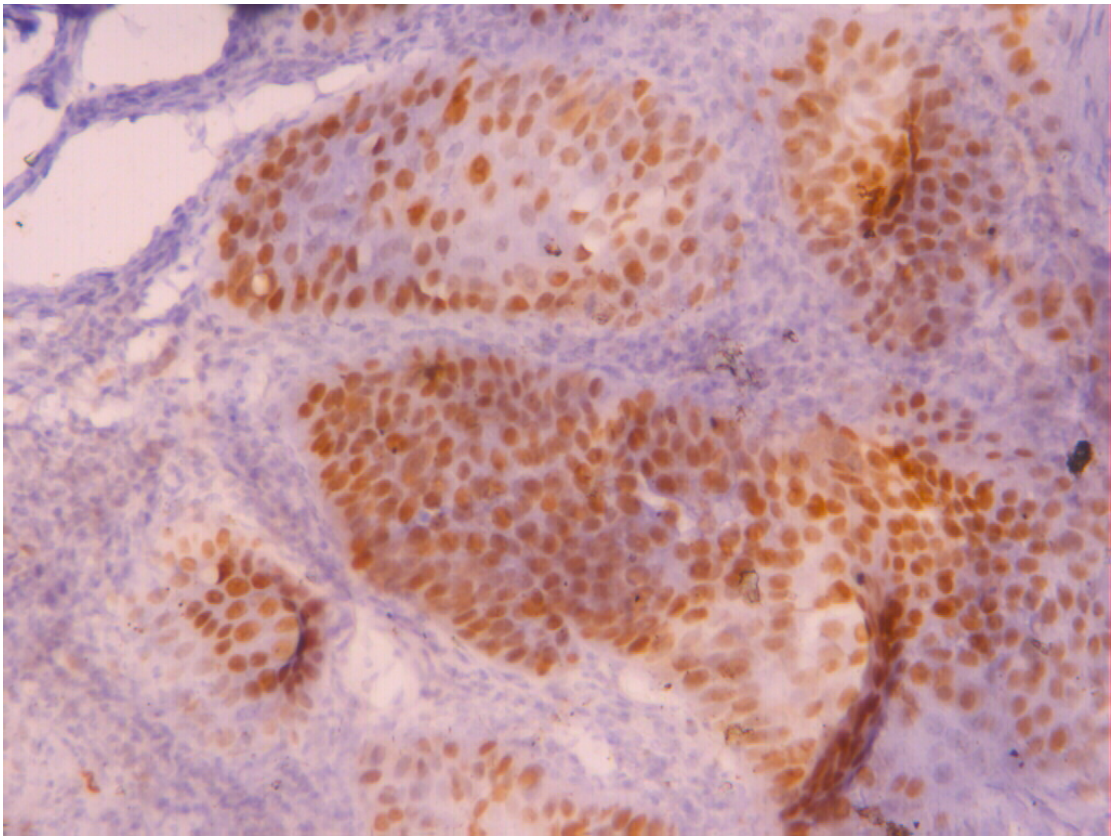

Figure 18: Photomicrograph of well differentiated OSCC- immune stain for p53. Counterstain used-hematoxylin. Islands of tumor cells with positive nuclear staining for p53. The deeply keratinized cells in the center of one of the island are negative for p53 staining.

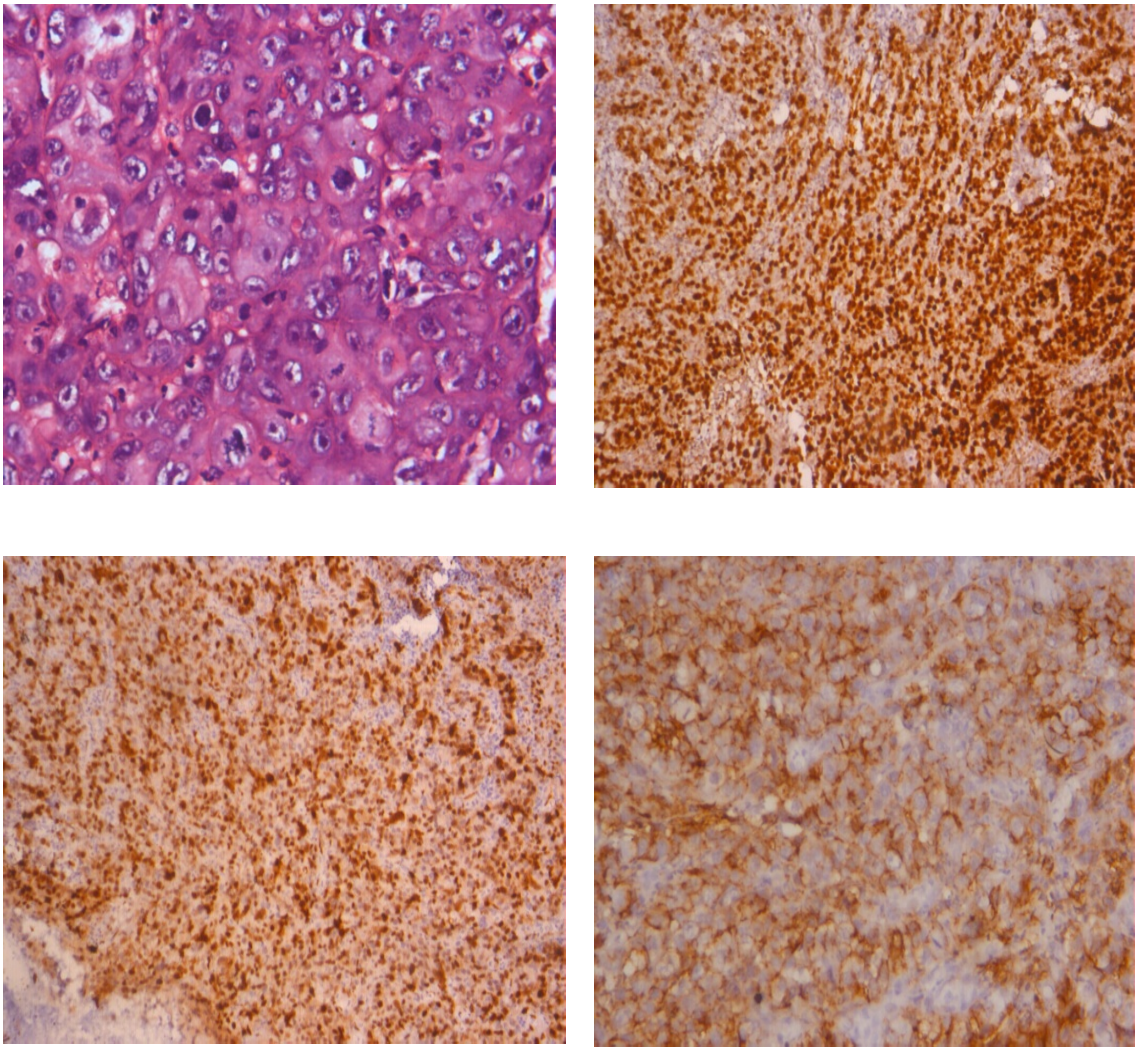

Figure 19: Photomicrograph of poorly differentiated SCC showing overexpression of all the 3 molecular markers p53, cyclin D1 and CD44. Upper left panel: H&E section showing markedly pleomorphic tumor cells. Upper right panel: Tumor cells showing strong positivity for p53 immunostaining. Bottom left panel: Tumor cells showing strong positivity for Cyclin D1 immunostaining. Bottom right panel: Tumor cells showing diffuse moderate positivity for CD44.

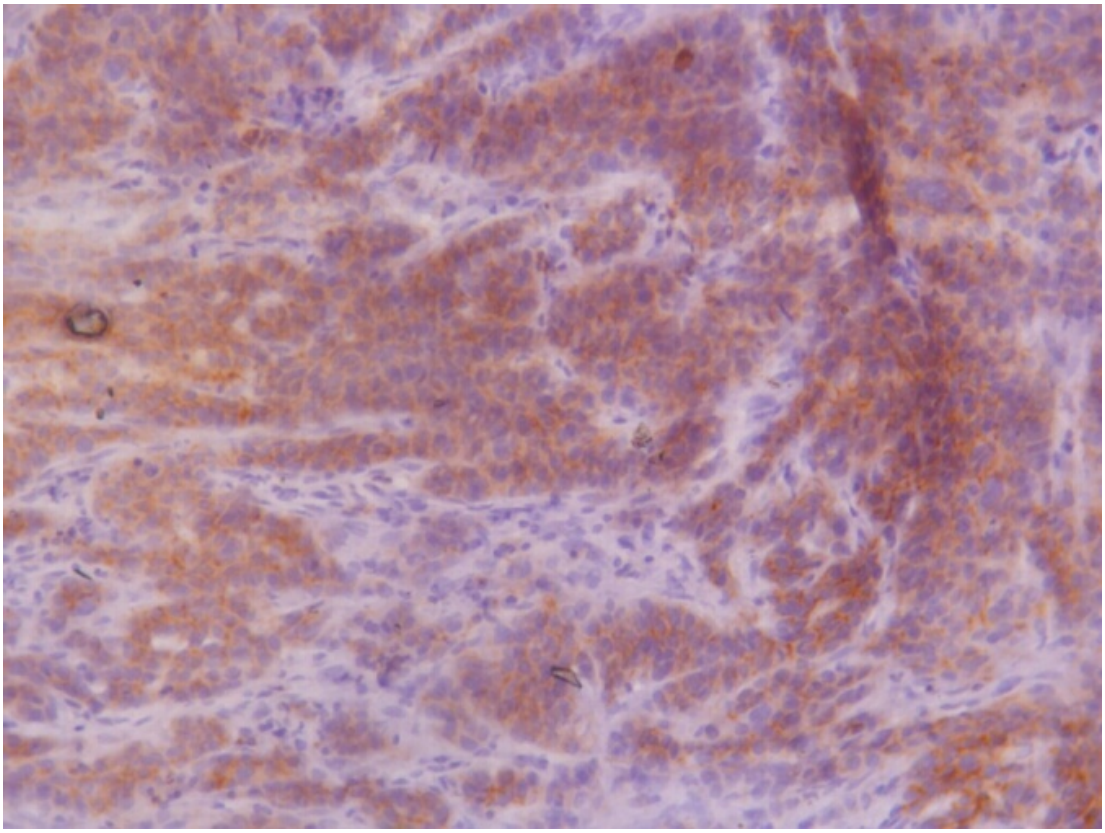

Figure 20: Photomicrograph of well differentiated oral squamous cell carcinoma with metastasis to regional lymph node. This picture shows moderate positivity for CD44 with a total score of 12.

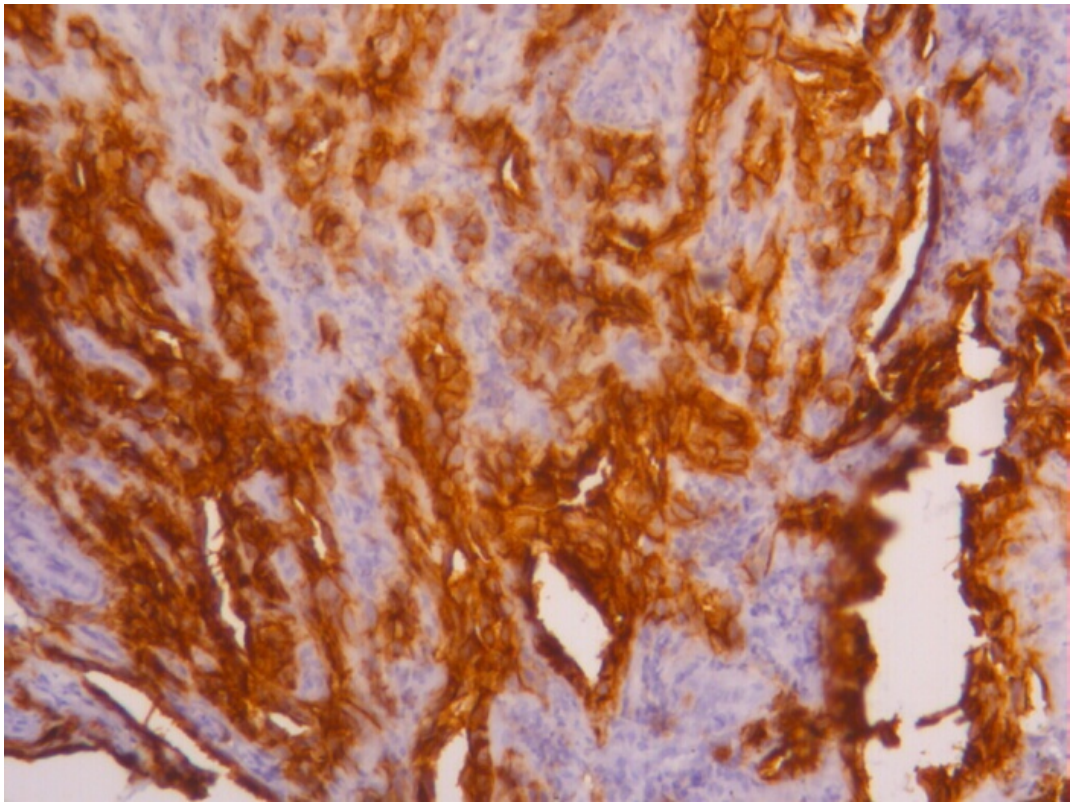

Figure 11: Photomicrograph of moderately differentiated oral squamous cell carcinoma with metastases to regional lymphnode. This picture shows diffuse strong membranous positivity for CD44 with a total CD44 score of 16.

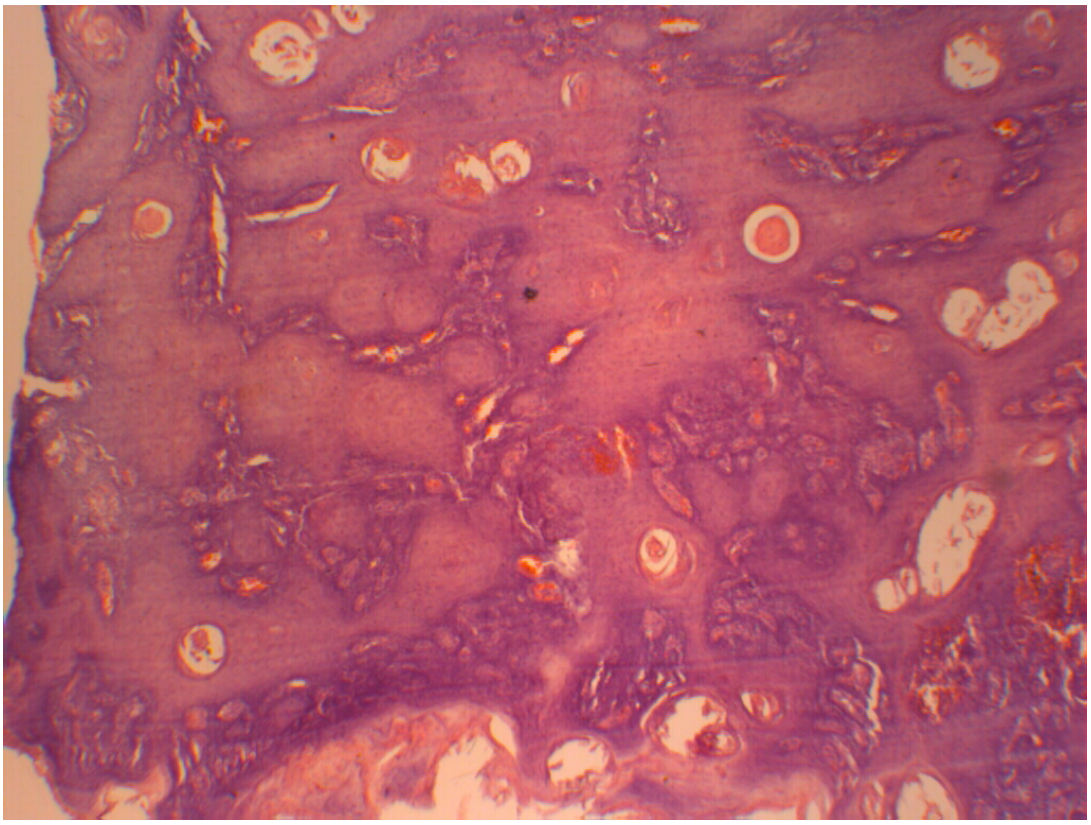

Figure 22: Photomicrograph of H & E stained section of a case of verrucous carcinoma (Magnification 40x). Note the intact basement membrane and many keratin pearls.

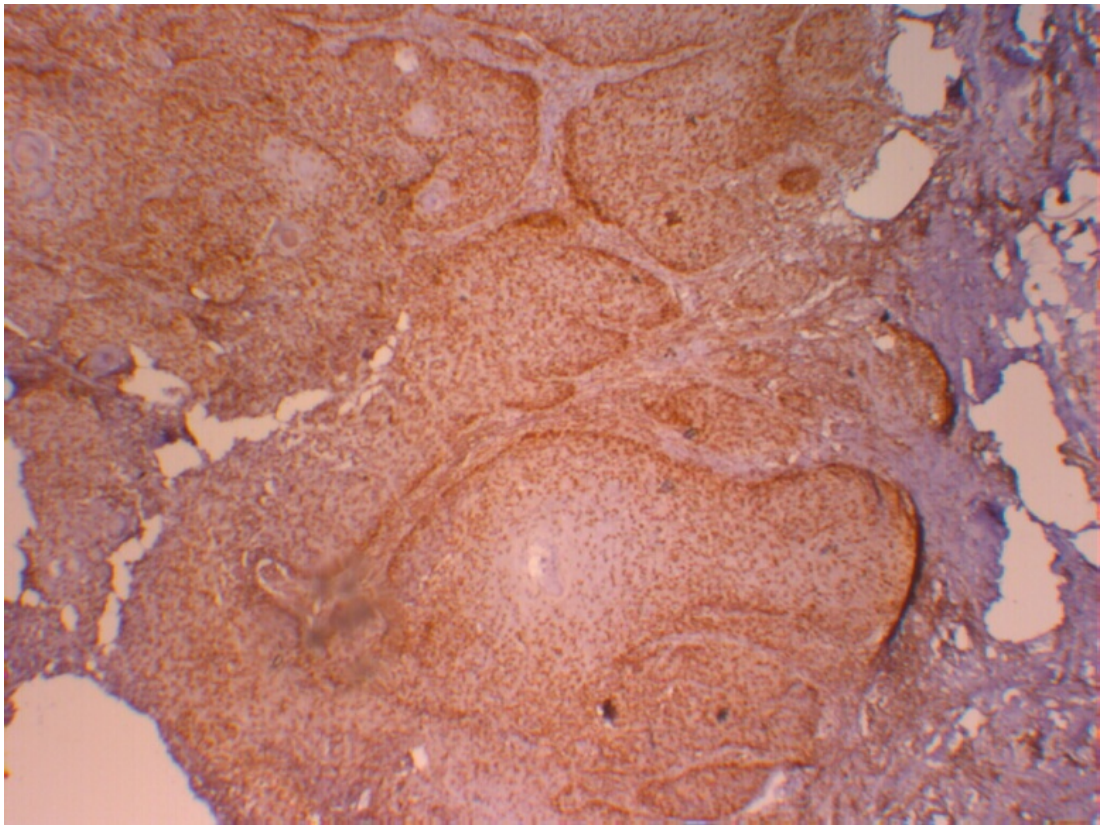

Figure 23: Photomicrograph of P53 immunohistochemical staining in a case of verrucous carcinoma (Magnification=40x). Tumor shows diffuse nuclear positivity for p53. Intensity of the staining appears to be strong in the periphery of the tumor islands when compared to the staining of cells in the center.

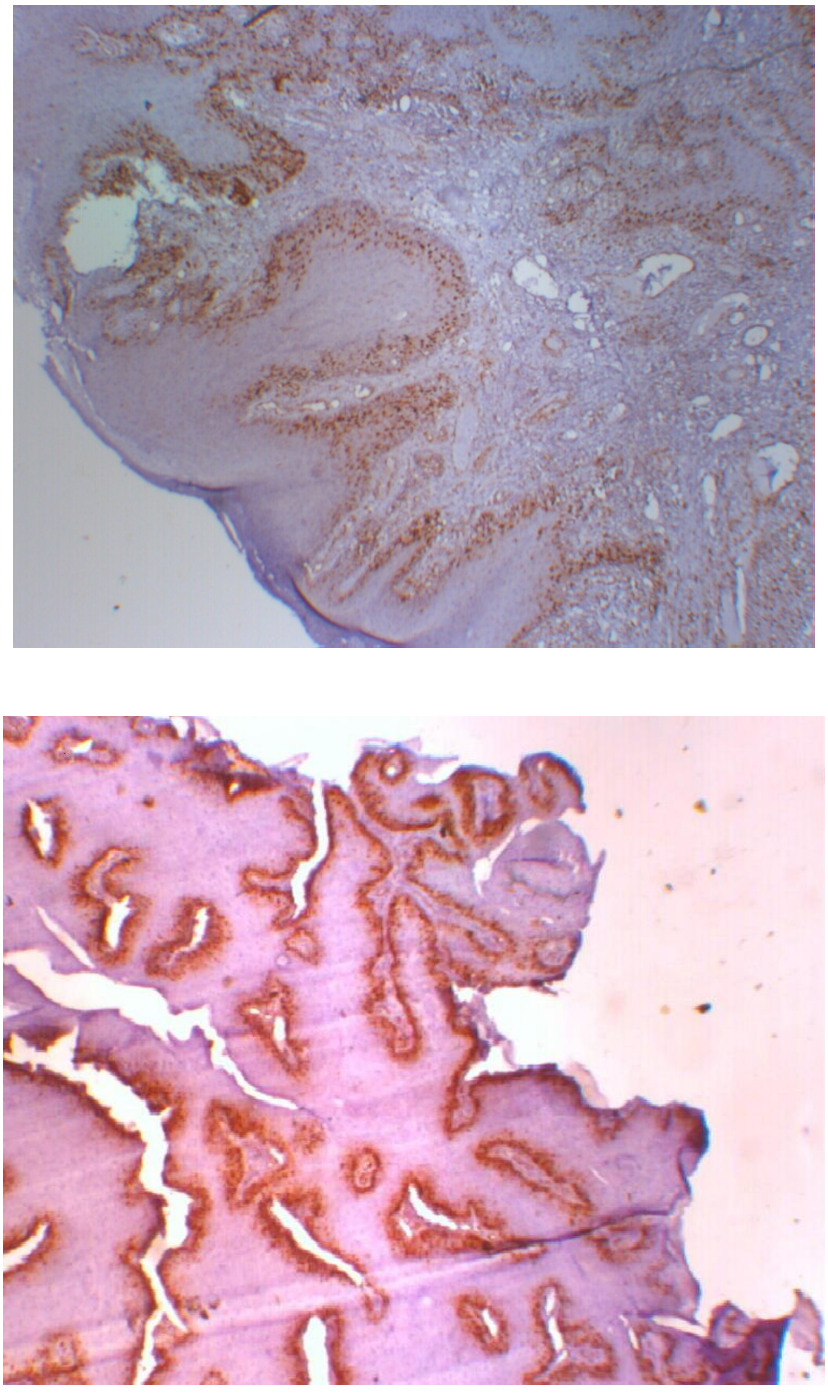

Figure 24: Photomicrograph of Cyclin D1 Immunohistochemical staining in two different cases of Verrucous carcinoma. Positive staining is seen in the nuclei of cells in the basal and suprabasal layer of the verrucous carcinoma.

APPENDIX I: PROFORMA

- Demographic Details
    - Name
    - Age (years)
    - Sex
- Identification Details
    - Ward
    - IP/OP No
- Chief complaints
- History of presenting illness
    - History suggestive of metastatic deposit
    - Bowel and micturition habits
    - Hypertension
    - Diabetes mellitus
- Risk Factors
    - Tobacco
    - Alcohol
    - Radiation exposure
    - Family history of OSCC

    - Immune defects
    - Occupational exposure
- Clinical Examination
    - Tumor Characteristics
        - Site
        - Nodal status
- Histopathological examination
    - Specimen type- Wegde biopsy/ Wide excision biopsy
    - Carcinoma type & grade
    - Adjacent epithelium
- Immunohistochemistry score for P53, CYCLIN D1 and CD44:
    - Extent (% positive tumor cells)
    - Intensity
    - Total score
    - Expression in adjacent epithelium
    - Intensity in adjacent epithelium

APPENDIX II: MASTER DATA SHEET

S.NO	AG	SE	WAR	ID NO	SP	HPE NO	FNAC NO	RISK FACTORS								SI	CA	ADJ	NO
								TOB	ALCO	HT	DM	RAD	FAM	IMM	OCC				
1	46	1	S3	205970	1	1023/08	NAP	1	1	0	0	0	0	0	0	4	5	1	NAP
2	55	1	S6	16626	1	108/09	NAP	1	1	1	0	0	0	0	0	2	3	0	0
3	50	2	ENT1	18775	1	119/09	F42/09	2	0	0	1	0	0	0	0	6	3	1	1
4	55	1	S	5636	1	120/09	F21/09	1	0	0	0	0	0	0	0	3	3	2	1
5	46	2	S6	250369	1	1369/08	NAP	2	0	0	0	0	0	0	0	2	2	0	0
6	58	1	ENT	NA	1	1601/09	NAP	1	0	0	0	0	0	0	0	8	4	1	0
7	45	1	S	NA	1	1604/09	NAP	1	1	0	0	0	0	0	0	6	4	0	0
8	65	1	ENT1	308943	1	1608/08	NAP	1	1	0	0	0	0	0	0	8	1	1	NAP
9	70	1	ENT2	46664	1	1943/08	NAP	1	1	0	1	0	0	0	0	5	3	0	0
10	68	1	ENT	437276	1	2328/08	F1857/08	3	1	0	0	0	0	0	0	7	3	1	1
11	80	2	S4	451683	1	2348/08	NAP	2	0	1	1	0	0	0	0	4	1	2	NAP
12	40	2	S1	107338	1	2379/08	F2093/08	2	0	0	0	0	0	0	0	5	3	2	1
13	47	2	S5	NA	2	2525/08	F1922/08	2	0	0	0	0	0	0	0	5	2	1	1
14	70	1	S3	484295	1	2581/08	F2051/08	3	0	0	0	0	0	0	0	4	2	1	1
15	63	1	S2	66961	1	2717/08	NAP	1	1	1	1	0	0	0	0	4	3	1	0
16	52	2	S	519373	1	2718/08	NAP	2	0	0	0	0	0	0	0	7	3	0	0
17	64	2	S	25968	1	2765/08	NAP	2	0	0	0	0	0	0	0	2	2	0	0
18	67	1	S6	450816	1	2798/08	NAP	2	1	0	1	0	0	0	0	5	2	1	0
19	40	2	S6	537448	1	2817/08	NAP	0	0	0	0	0	0	0	0	1	5	1	NAP
20	43	1	S	540819	1	2827/08	NAP	0	1	0	0	0	0	0	0	4	3	1	0
21	53	1	ENT	14902	1	644/08	F561/08	1	1	1	0	0	0	0	0	5	2	1	1
22	60	2	S5	8838	1	66/09	NAP	2	0	1	0	0	0	0	0	1	3	1	0
23	70	2	S3	1799	1	763/08	NAP	2	0	1	0	0	0	0	0	5	2	0	0
24	70	2	S5	17316	2	857/08	NAP	2	0	0	0	0	0	0	0	4	5	1	NAP
25	50	2	S	16537	1	926/08	NAP	2	0	0	0	0	0	0	0	1	5	1	NAP

S.NO	P53							CYCLIND1							CD44						
	EXP	EXT	IN	TS	TS≥4	EXPA	INA	EXP	EXT	IN	TS	TS≥4	EXPA	INA	EXP	EXT	IN	TS	TS>4	EXPA	INA
1	1	2	3	5	1	1	4	1	2	3	5	1	3	3	1	3	3	9	1	3	3
2	1	3	3	6	1	NAP	NAP	1	2	3	5	1	NAP	NAP	0	1	1	1	0	NAP	NAP
3	1	3	2	5	1	3	3	1	3	2	5	1	2	2	0	1	1	1	0	3	3
4	1	3	3	6	1	4	4	1	1	3	4	1	4	4	1	2	2	4	0	4	4
5	1	2	2	4	1	NAP	NAP	1	1	1	2	0	NAP	NAP	0	1	1	1	0	NAP	NAP
6	1	4	3	7	1	1	3	1	3	3	6	1	2	4	1	4	4	16	1	3	4
7	1	4	3	7	1	NAP	NAP	1	1	2	3	0	NAP	NAP	1	2	2	4	0	NAP	NAP
8	1	4	3	7	1	3	4	1	4	3	7	1	3	4	1	4	4	16	1	3	3
9	1	3	2	5	1	NAP	NAP	1	1	2	3	0	NAP	NAP	1	2	3	6	1	NAP	NAP
10	1	4	3	7	1	3	3	1	4	3	7	1	3	4	1	3	3	9	1	3	1
11	1	4	3	7	1	3	4	1	1	3	4	1	4	4	1	2	3	6	1	4	4
12	1	3	3	6	1	3	4	1	3	3	6	1	4	4	1	4	4	16	1	4	4
13	1	3	3	6	1	3	3	1	2	3	5	1	2	4	0	1	1	1	0	3	3
14	1	3	3	6	1	3	3	1	3	3	6	1	2	4	1	2	2	4	0	3	4
15	1	2	1	3	0	1	2	1	2	2	4	1	2	3	1	3	3	9	1	3	3
16	1	3	3	6	1	NAP	NAP	1	3	3	6	1	NAP	NAP	1	3	3	9	1	NAP	NAP
17	1	3	2	5	1	NAP	NAP	1	2	2	4	1	NAP	NAP	1	3	4	12	1	NAP	NAP
18	1	2	1	3	0	1	2	1	2	2	4	1	2	3	1	3	4	12	1	3	3
19	1	2	3	5	1	1	3	1	2	3	5	1	2	3	1	3	4	12	1	1	4
20	1	3	1	4	1	1	4	1	2	2	4	1	2	4	1	3	3	9	1	3	2
21	1	3	3	6	1	3	4	1	3	2	5	1	2	4	1	4	3	12	1	3	3
22	1	4	3	7	1	1	4	1	3	3	6	1	2	4	1	3	4	12	1	3	3
23	1	3	3	6	1	NAP	NAP	1	1	1	2	0	NAP	NAP	1	2	2	4	0	NAP	NAP
24	1	3	3	6	1	1	4	1	2	2	4	1	2	4	1	3	3	9	1	3	4
25	1	2	3	5	1	1	4	1	2	3	5	1	3	4	1	3	3	9	1	3	1

SEX: 1-Male, 2-Female. **SPECIMEN:** 1-Wedge Biopsy, 2-Excision Biopsy. **TOBACCO:** 1-Smoking, 2-Chew, 3-Snuff. **SITE:** 1-Lip, 2-Alveolar Margin; 3-Floor Of The Mouth; 4-Buccal Mucosa; 5-Tongue; 6-Retromolar Trigone; 7-Palate; 8-Tonsil. **CANCER GRADE AND TYPE:** 1-Insitu; 2-Well Differentiated; 3-Moderately Differentiated; 4-Poorly Differentiated; 5-Verrucous Carcinoma. **ADJ EPI:** 0-No Adjacent Epithelium Available; 1-Normal; 2-Dysplastic. **NODAL STATUS:** 0-Absent; 1-Present; Nap-Not Applicable. **P53 EXT IN CA:** 0-No Cells; 1-<10%; 2- 10 T0 50%; 3-50 To 90%; 4->90%. **P53 INT IN CA:** 0-Absent; 1-Equivocal; 2-Clearly Positive; 3-Strongly Positive. **P53 EXP ADJ:** 0-Absent; 1-Present in Basal Layer; 2-Present in Suprabasal Layer; 3-Present in Both Basal and Suprabasal Layer; 4- Present in Dysplastic Epithelium. **P53 INT IN ADJ EPI:** 1-Weak; 2-Mild; 3-Moderate; 4-Strong. **CYD1 EXT IN CA:** 0-No Cells; 1-<10%; 2- 10 T0 50%; 3-50 To 90%; 4->90%. **CYD1 INT IN CA:** 0-Absent; 1-Equivocal; 2-Clearly Positive; 3-Strongly Positive. **CYD1 EXP ADJ:** 0-Absent; 1-Present in Basal Layer; 2-Present in Suprabasal Layer; 3-Present in Both Basal and Suprabasal Layer; 4- Present in Dysplastic Epithelium. **CYD1 INT IN ADJ EPI:** 1-Weak; 2-Mild; 3-Moderate; 4-Strong. **CD44 INT IN CA:** 1-None; 2-Mild; 3-Moderate; 4-Strong. **CD44 EXP ADJ:** 0-Absent; 1-Present in Basal Layer; 2-Present in Suprabasal Layer; 3-Present in Both Basal and Suprabasal Layer; 4- Present in Dysplastic Epithelium. **CD44 INT IN ADJ EPI:** 1-Weak; 2-Mild; 3-Moderate; 4-Strong.

APPENDIX III: DETAILS OF THE REAGENTS USED IN IMMUNOHISTOCHEMICAL ANALYSIS

Primary Antibody	Name	Anti p53 Antibody	Anti Cyclin D1 Antibody	Anti CD44 Antibody*
	Clone	D07; Mouse monoclonal	Not Available; Rabbit	DF1485; Mouse monoclonal
	Supplier	Bio Genex	Bio Genex	Bio Genex
	Catalog Number	Not Available (6ml ready to use antibody)	AR4447-5R; (6ml ready to use antibody)	AM310-5M (6ml ready to use antibody)
	Dilution	-	-	-
	Incubation Time/Temp	60 min/room temperature	60 min/room temperature	120 min/room temperature
Antigen Retrieval	Device	Microwave Oven	Microwave Oven	Microwave Oven
	Buffer/pH value	Tris EDTA / 9	Tris EDTA / 9	Citrate / 6
	Heat Temp/Time	Medium 10 mts, high 10 mts followed by medium 10 mts	Medium 10 mts, high 10 mts followed by medium 10 mts	Medium 10 mts, high 10 mts followed by medium 10 mts
	Cool Temp/Time	Room temp/20 minutes	Room temp/20 minutes	Room temp/20 minutes
Detection Methods		Polymeric method	Polymeric method	Polymeric method
Chromogen Substrate	Reagent	DAB	DAB	DAB
	Incubation Time/Temp	5-8 minutes/room temp	5-8 minutes/room temp	5-8 minutes/room temp
Counterstain	Reagent	Ehrlich's Hematoxylin	Ehrlich's Hematoxylin	Ehrlich's Hematoxylin
	Staining Time	30 seconds	30 seconds	30 seconds
Results	Staining Pattern	Nucleus	Nucleus	Cell membrane
Additional Information	Species Reactivity	Porcine	Porcine	Porcine
	Fixation	Formalin fixed paraffin sections	Formalin fixed paraffin sections	Formalin fixed paraffin sections
	Blocking	2-5% normal serum to reduce unspecific background staining;0.5-3% H_2O_2 to block endogenous peroxidase activity		

* - The monoclonal antibody to total CD44.

APPENDIX IV: IMMUNOHISTOCHEMISTRY PROCEDURE

1. Sections were cut at 4 microns, taken in a coated slide & incubated at 58°C overnight.
2. Deparaffinized in Xylene for 30 minutes.
3. Immersed in absolute alcohol for 2 minutes X 2 changes.
4. Washed in tap water for 10 minutes.
5. Rinsed in distilled water for 5 minutes.
6. Antigen retrieval was done using microwave sequentially- 4 times: Initially at medium energy for 10 minutes followed by high energy for 10 minutes and finally at medium energy for 5 minutes twice.
7. Cooled to room temperature for 20 minutes.
8. Rinsed in distilled water for 5 minutes.
9. Washed in TBS wash buffer for 5 minutes X 2 changes.
10. The sections were covered with peroxide block for 10 minutes.
11. Washed in TBS wash buffer for 5 minutes X 2 changes.
12. The sections were covered with power block for 10 minutes.
13. The sections were drained and covered with primary antibody without washing (p53 and Cyclin D1 for 1 hour; CD 44 for 2 hours) and kept in a moisture chamber.
14. Washed in TBS wash buffer 5 minutes X 2 changes.
15. The sections were covered with super enhancer for 30 minutes.
16. Washed in TBS wash buffer for 5 minutes X 2 changes.
17. The sections were covered with S.S label + poly HRP for 30 minutes.
18. Washed in TBS wash buffer for 5 minutes X 2 changes.
19. The sections were covered with chromogen (DAB + substrate buffer) for 5 to 8 minutes.
20. Washed in TBS wash buffer 5 minutes X 2 changes.
21. Washed in tap water for 5 minutes.
22. Counter stained with hematoxylin for 30 seconds.
23. Washed in tap water for 5 min.
24. Air dried, cleaned in Xylene and mounted with DPX mountant.

RESULT: The development of brown color was interpreted as positive and scoring was done using the method mentioned previously.

Note: The sections should not be allowed to dry throughout the staining procedure.