

## Immunohistochemistry examination to reveal the pathogenesis of Oral Squamous Cell Carcinoma

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**Abstract.** Oral mucosal cancer is a type of cancer that develops from the lining of the oral cavity (mucosa). The main risk factors are smoking and drinking alcohol. The pathogenesis of oral mucosal cancer involves various interrelated etiologies such as smoking and alcohol consumption, human papilloma virus (HPV), and patients who have undergone hemopoietic stem cell transplants (stem cell transplants). Meanwhile, it does not rule out the possibility of internal factors such as genetics. There are several types of oral mucosal cancer, but oral squamous cell carcinoma is the most common type of oral cancer and represents more than 90% of all head and neck cancers. Immunohistochemical examination of the Oral squamous cell carcinoma smear biopsy material which included examination of antibodies in the form of cytokeratin, CDT1, Ki-67, Geminin, Mcm2,  $\gamma$ -H2A/ histone family member X and Aurora-A, BCL-2, VEGF, p53, p63, p73, Prb, c-erbB2 or HER2/neu, upregulation of telomerase (human telomerase reverse transcriptase; hTERT), loss of heterozygosity (Chromosome loci 3p, 8p, 9p, 4q, 11q, 13q, 17p), High-risk Human papillomavirus 16/18 (HR-HPV16 and 18), p16, Overexpression of EGFR, c-jun, c-fos, surviving (BIRC5), MMP-9, MMP9, TGF-, COX-1, and-2, and amplification of Cyclins D and E. Thus, paving the way for appropriate therapy.

**Keyword:** Immunohistochemistry, pathogenesis of Oral Squamous Cell Carcinoma

**Abstrak.** Kanker mukosa mulut adalah jenis kanker yang berkembang dari lapisan rongga mulut (mukosa). Faktor risiko utama adalah merokok dan minum alkohol. Patogenesis kanker mukosa mulut melibatkan berbagai etiologi yang saling terkait seperti merokok dan konsumsi alkohol, human papilloma virus (HPV), dan pasien yang telah menjalani transplantasi sel induk hemopoietik dan tidak menutup kemungkinan adanya factor genetik. Ada beberapa jenis kanker mukosa mulut, tetapi karsinoma sel skuamosa rongga mulut adalah jenis kanker mulut yang paling umum dan mewakili lebih dari 90% dari semua kanker kepala dan leher. Pemeriksaan imunohistokimia bahan biopsi smear Oral squamous cell carcinoma meliputi pemeriksaan antibodi berupa sitokeratin, CDT1, Ki-67, Geminin, Mcm2, -H2A/ anggota keluarga histone X dan Aurora-A, BCL-2, VEGF, p53, p63, p73, Prb, c-erbB2 atau HER2/neu, upregulasi telomerase (human telomerase reverse transcriptase; hTERT), hilangnya heterozigositas (Kromosom lokus 3p, 8p, 9p, 4q, 11q, 13q, 17p), Human papillomavirus 16/18 (HR-HPV16 dan 18), p16, Overekspresi EGFR, c-jun, c-fos, bertahan

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*(BIRC5), MMP-9, MMP9, TGF-, COX-1, dan-2, dan amplifikasi Cyclins D dan E. Dengan demikian, penemuan-penemuan ini membuka jalan untuk terapi yang tepat.*

**Kata Kunci:** *Imunohistokimia, Patogenesis Karsinoma Sel Skuamosa Rongga Mulut*

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## 1 Introduction

Oral cancer, also called oral cancer, is a cancer that attacks the mucosal epithelial tissue in the oral cavity (including the lips, gums, floor of the mouth, tongue, cheeks, and palate). Initially, this cancer certainly did not grow right away, but was preceded by the appearance of sores in the mouth, which at first glance resembled canker sores but did not heal. Oral cancer is rarely detected early. Most cases of this disease are usually found after the spread to the lymph nodes in the neck. There are many types of cells in the oral cavity and the throat, so the types of cancer are divided into several types, including:

- Squamous cell carcinoma. More than 90% of oral cancers are squamous cell carcinomas, which attack the flat cells that line the mouth and throat.
- Verrucous carcinoma. Less than 5% of oral cancers are verrucous carcinomas that develop slowly and rarely affect other parts of the body. This type of cancer can form from squamous cell carcinoma that gets worse.
- Other types of cancer. Minor salivary gland carcinoma and lymphoma are types of oral cancer that are quite rare. This cancer forms in the glands in the lining of the mouth and throat, tonsils, and base of the tongue.

According to Globocan data in 2020 [1], cancer of the tongue, gums, and lips ranks 17th as the most common type of cancer in Indonesia. It is known that the latest number of cases reached 5.780 people, with a death toll of 3.087.

Immunohistochemical examination is an examination technique using antibodies to specifically detect the presence of certain proteins that act as antigens in cells. This check is superior to immunological examination serum because it can indicate the type of cell and damaged tissue that express antigenic proteins and can detect the sites of antigens in both lesion and normal tissue [2]. Immunohistochemical examination with antibodies to various proteins expressed can reveal the pathogenesis of oral mucosal cancer, so that every patient can receive individualized therapy. Understanding the pathogenesis of oral mucosal cancer is necessary because it is closely related to management.

The biopsy technique requires a representative portion of the lesion and a margin of normal tissue. The biopsy can be done by incisional or excisional. Incisional biopsy is chosen if the surface lesion is large (more than 1 cm) and excisional biopsy, i.e., an intoto-total incision, if the lesion

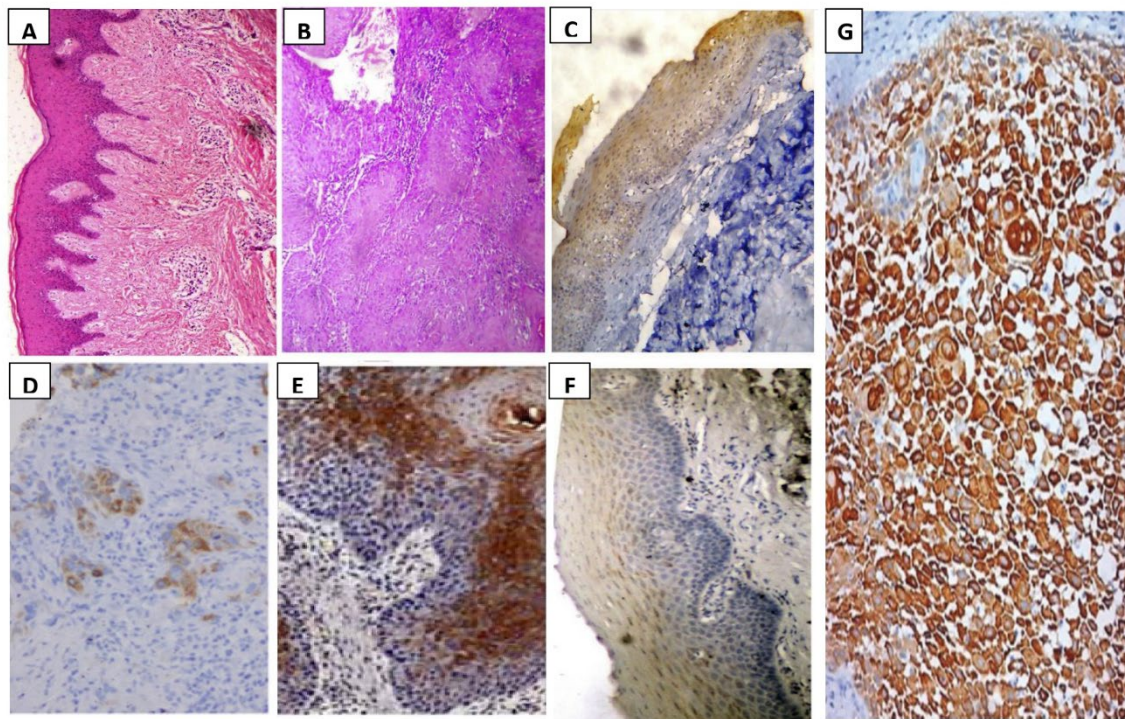
is small [3]. An incisional or excisional biopsy is performed by making an incision in the skin prior to tissue sampling. The length of the slice size depends on the need and availability of the biopsy technique. An excisional or open-slice biopsy is performed if a large sample is required. After the biopsy is done, the incision will be closed using stitches. The skin biopsy material was put in 10% formalin fixation and paraffin blocks were made, which then, after being cut with a microtome, skin biopsy specimens were obtained on an object glass. Many microscopic specimens can be cut from a single block of paraffin, and each specimen can then be immunohistochemically examined with antibodies that match the antigen being examined. To detect one type of antigen, antibodies that are specific to that antigen are needed. One conventional immunohistochemical examination can only detect one type of antigen, so if it is necessary to detect 2 types of antigens, 2 examinations are required, which can be taken from one skin biopsy material [2]. Another alternative is skin biopsy material without formalin fixation (fresh tissue) frozen and cut with a cryostat and then examined with antibodies labeled with fluorescent material, known as immunofluorescent examination. The following are various types of immunohistochemical (and immunofluorescent) tests that can be performed to reveal the pathogenesis of oral squamous cell carcinoma [4].

## **2 Immunohistochemical examination with cytokeratin antibodies (CK1, CK5, CK7, CK8 and CK18, CK19)**

Oral squamous cell carcinoma (OSCC) may be preceded by potentially malignant disorders such as leukoplakia and oral submucous fibrosis (OSF) and has a greater than normal risk of malignant transformation [5]. This examination was carried out to evaluate and compare the expression of CK1, CK5, CK7, CK8 and CK18 and CK19 in normal oral mucosa OSCC by immunohistochemistry. Cytokeratins (CKs) are a group of intermediate filament proteins dispersed in the cytoplasm of eukaryotic cells that contribute to the maintenance of the cytoskeletal framework of these cells and are specifically expressed by epithelial tissues. Cytokeratins (CKs) are a group of intermediate filament proteins dispersed in the cytoplasm of eukaryotic cells that contribute to the maintenance of the cytoskeletal framework of these cells and are specifically expressed by epithelial tissues [6]. CKs are broadly classified on the basis of their molecular weight and isoelectric points into 2 subfamilies: type I: acidic with a low molecular weight (40–64 kDa), CKs 9–23; and type II: basic or neutral with a high molecular weight (52–68 kDa). CKs 1–8. CK1, CK5, CK7, CK8, CK18, and CK19 are the most common and characteristic members of the large intermediate filament gene family and are expressed in "simple" or single-layer epithelial tissues [7].

Based on the results of Nanda et al. [8], they found that the present study found no expression of CK8 and CK18 in normal buccal mucosa and an increased intensity of expression in OSCC. Meanwhile, CK8 staining was detected in 20% and CK18 in 40% of the leukoplakia in the present study, which was consistent with the studies done by Vaidya et al. in [9] and Vigneswaran et al.

in [10]. Detection of CK1, CK5, CK7, CK19 was found in the previous studies of Sharada, et al. in [11], Prabakaran, et al. in [6], Rajeswari, et al. in [12], and Gurda, et al. in [13]. The result of their study was the prognostic implications of loss of CK5 and gain of CK1, CK7, CK8, CK18, and CK19 in oral potentially malignant lesions and squamous cell carcinomas. Altered CK1, CK5, CK7, CK8, CK18, and CK19 expression patterns could be an early event in the pathogenesis of OSCC. Therefore, CK8 and CK18 have potential use as surrogate markers of malignant transformation. Figure 1 shows the results of several previous studies regarding the detection of cytokeratin proteins on the prognosis of OSCC and compared with staining using HE.



**Figure 1.** shows representative photomicrographs of [A] normal oral squamous cells stained with HE, [B] OSCC stained with HE, [C] OSCC stained with IHC for CK8, [D] OSCC stained with IHC for CK5, [E] OSCC stained with IHC for CK1, [F] OSCC stained with IHC for CK18, and [G] OSCC stained with IHC for CK19. Nanda et al. in [8], Sharada et al. in [11], Prabakaran et al. in [6], Rajeswari et al. in [12], and Gurda et al. in [13] provided the image.

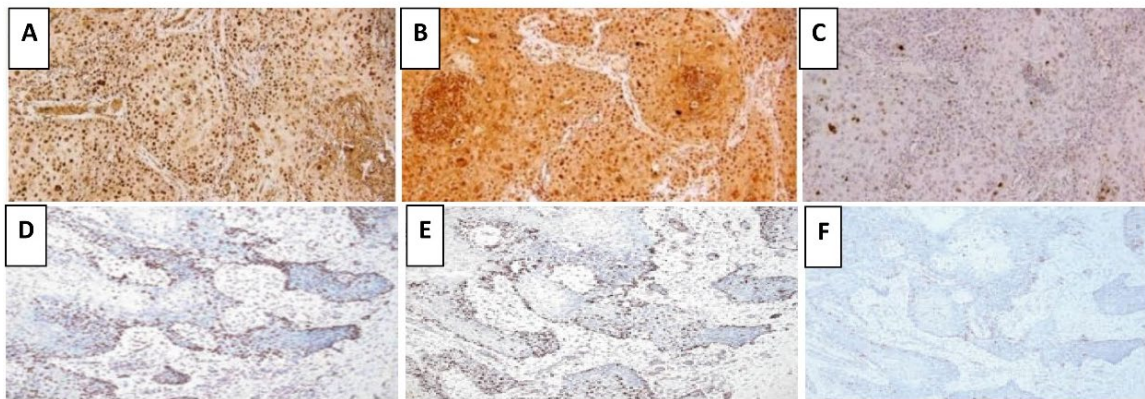
### 3 Immunohistochemical examination with Chromatin licensing and DNA replication factor 1 (CDT1), Ki-67, Geminin, Mcm2, $\gamma$ -H2A/ histone family member X and Aurora-A

Proteins necessary for the normal regulation of the cell cycle include minichromosome maintenance protein 2 (Mcm2) and geminin. These are overexpressed in several premalignant and malignant tumours. The Mcm2/Ki67 ratio can be used to estimate the population of cells that are in early G1 (licensed to proliferate), and the geminin/Ki67 ratio can determine the relative length of G1. A high ratio indicates a short G1 and a high rate of cell proliferation. Mcm2 and geminin have been scarcely explored in oral epithelial dysplasia (OED) and OSCC, but they are very necessary for early detection of OSCC [14]. Meanwhile, from the riset of Al-Hazmi, et al. in [15], they found a link between MCM2, Ki67, and geminin expression and tumor histology



and invasive front grade (P 0.05). Aneuploid DNA was found in 82% of the OSCC studied, which was linked to increased Aurora-A expression intensity (P = 0.01). TNM staging was associated with geminin and the geminin/Ki67 ratio (P 0.05), and low expression of MCM2, Ki67, geminin, and Aurora-A was predictive of OSCC survival (P 0.05). Understanding tumor cell-cycle kinetics can help with diagnosis, prognosis, and targeting cell-cycle phase-specific agents. MCM2 and Ki67 expression is constant across all cell-cycle phases (G1, S, G2, and M). However, Ki67 is only expressed in proliferating cells, whereas MCM2 may be expressed in non-proliferating cells during the G0 phase [16, 17, 18].

Clinically, pKi67 has been shown to correlate with tumor metastasis and clinical stage according to Li, et al. in [19]. Furthermore, it has been demonstrated that Ki67 expression is significantly higher in malignant tissues with poorly differentiated tumor cells than in normal tissue. pKi67 expression, through its predictive role, identifies subpopulations of patients who are more likely to respond to a given therapy. The Ki67 labeling index, which includes all stages and grade categories, is an independent prognostic factor for the survival rate. There is a link between the proportion of Ki67+ malignant cells and patient survival. Meanwhile, in Siril, et al. in [18], the same discovery was reported. The prickly cell layer of oral squamous cell carcinoma expressed chromosome licensing and DNA replication factor 1. Geminin reactivity was found to be widespread in oral squamous cell carcinoma.  $\gamma$ -H2A histone family member X was found in small amounts in oral squamous cell carcinoma. As a result, geminin and  $\gamma$ -H2A histone family member X were linked to differentiation and invasion mode. Here are the findings from several previous studies on CDT1, Ki-67, Geminin, Mcm2,  $\gamma$ -H2A/ histone family member X and Aurora-A (Figure 2).



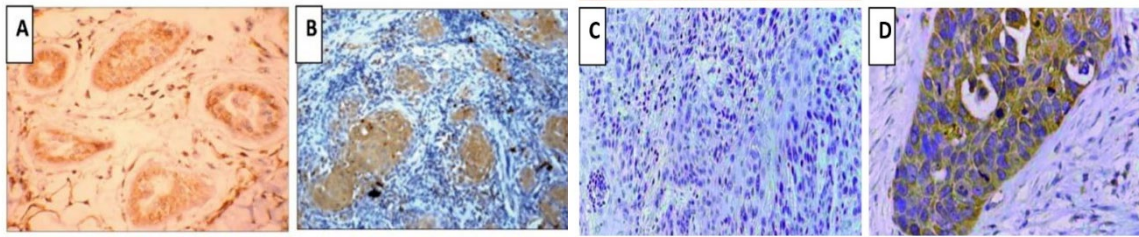
**Figure 2.** Representative photomicrographs of [A] CDT1, [B] Geminin, [C]  $\gamma$ -H2A, [D] Mcm2, [E] Ki67, and [F] Aurora-A by IHC staining. [picture was taken from Al-Hazmi, et al. in [15] and Siril, et al. in [18].

#### **4 Immunohistochemical examination with Apoptosis Marker BCL-2, Inhibitor of Apoptosis Survivin (BIRC5)**

BCL-2 proteins are among the most abundant anti-apoptotic proteins found in OSCC. The majority of studies suggest that Bcl-2 expression could be used as a prognostic indicator for OSCC. They aid in the development of cancer and mediate resistance to current anticancer treatments. New cross-sectional retrospective studies show that bcl-2 upregulation is the first step in epithelial karsinogenesis. OSCC carcinogenesis is a multistage process involving oncogene activation and inactivation of tumor suppressor genes with an imbalance of cell death and growth [20]. Based on the research by Popovic et al. in [21] got a low intensity of BCL-2. A low level of bcl-2 expression in their sample appears to be associated with a higher survival rate: 77% over a 5-year follow-up period, implying that bcl-2 expression could be a valuable predictor of tumor behavior and disease outcome. The same report, found in Juneja et al [22], about BCL-2, In OSCC, Bcl-2 immunoreactivity was prominent in the peripheral cells of the infiltrating tumor islands, which diminished toward the center in well-differentiated and moderately differentiated OSCC, whereas stronger and more diffuse expression of Bcl-2 oncoprotein was seen in poorly differentiated OSCC. In their study, OSCC had an overall positivity of 30% (9/30). Although the expression of BCL-2 was found in all the research investigated, some of them found the expression of BCL-2 in a different part of the cell. Singh et al.,[23] Yao et al., [24], Loro et al., [25], and Solomon et al. [26] demonstrated that Bcl-2 oncoprotein is observed as cytoplasmic granular staining.

In addition to the Bcl-2 and p53 families, proteins that play a role in regulating the balance of apoptosis, cytokinesis, and signal transduction are IAPs. The appearance of this protein was triggered by the presence of the Baculovirus IAP Repeat (BIR) domain protein. Currently, there are eight IAPs that have been identified: NAIP (BIRC1), cIAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/MLIAP (BIRC7), and IAP-like protein 2 (BIRC8) [27]. These proteins are endogenous caspase inhibitors, which act by how they bind to the BIR domain on the active site of caspase, resulting in caspase degradation, or keeping caspases away from the substrate. Disruption of IAPs regulation has been widely reported in some cases cancer [28]. survivin, closely related to OSCC. Expression of survivin can be used as a marker of OSCC aggressiveness in high-risk patients [29]. Broadly speaking, caspases are divided into two groups, namely those related to caspase-1 and those involved in caspase-1 in the release of cytokines during the inflammatory process (caspase-1, 4, 5, 13, and 14). 39 The second group is the ones that most play a role in apoptosis (caspase-2, 3, 6, 7, 8, and 9) [30]. This second group is divided into two parts, namely caspase initiators (caspase-2, 8, 9, and 10) that play a role in the pathway of early apoptosis and effector caspases (caspase-3, 6, and 7), which play a role in the leakage of cellular components during apoptosis [30]. Li et al. [31] showed a decrease in the activity of caspase-3 zymogen and increased survivin expression in KSSM tumor tissue. This study describes survivin, which inhibits the synthesis of

and activation of caspase-3, thereby inhibiting cell apoptosis KSSM [32]. The expression of BCL-2 and survivin in OSCC in different parts of the cells is shown in figure 3.



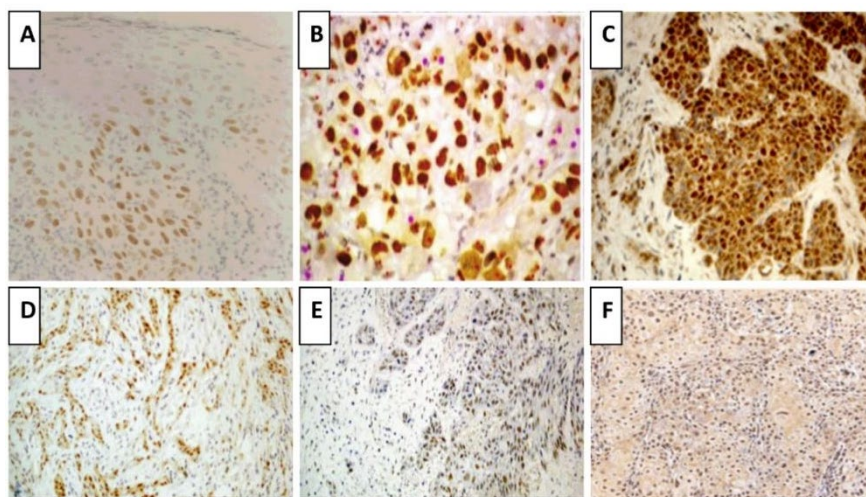
**Figure 3.** Representative photomicrographs of [A] Bcl-2 positivity in peripheral cells and diminishing toward the center of the epithelial islands in well-differentiated squamous cell carcinoma ( $\times 400$ ), [B] Overexpression of Bcl-2 in the cytoplasm and nuclear envelop of tumor cells. (IHC-40 $\times$ ), [C] A case of moderately differentiated oral SCC with no survivin staining, score 0 (ABC, 150 $\times$ ), [D] A case of low-grade differentiation oral SCC with cytoplasmic positivity for survivin at high power, score 4 (ABC, 400 $\times$ ), [picture was taken from Lo, et al. in [33] and Juneja, et al. in [22].

## 5 Expression of tumor cell markers and protooncogenes p53, p63, p73, c-jun, c-fos, and hTERT.

Carcinogenesis is a multi-step process that begins with the loss of the arms of chromosomes 3p and 9p. This condition occurs when normal tissue changes to dysplasia [34]. It is followed by the loss of several other chromosomal arms, such as 8p, 13q, and 17p. Loss of tumor suppressor genes is suspected to occur at sites where p16 loss occurs at 9p and the gene p53 is missing at 17p [45]. The presence of cellular stress increases the expression of the protein p53, which results in G1 arrest or apoptosis. ASPP 1 and ASPP 2 are members of the Apoptosis Stimulating Protein p53 (ASPP) family. They specifically stimulate p53 transactivation in promoters of pro-apoptotic genes such as Bax and p53 inducible gene 3 (PIG 3), but not in promoters of cell cycle inhibitor genes such as p21 and mdm2 [36].

The p53 gene not only functions in apoptosis but also plays an important role in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, DNA segregation, and cellular aging, so it is called the "Guardian of the genome". Damage to the p53 tumor suppressor gene has been detected in more than 50% of the total percentage of cancer incidence [37]. Research conducted by Cruz et al. in [38] also showed p53 expression in 86% of cases of premalignant lesions above the -cell layer basal layer that are likely to develop into KSSM. Motta et al. in [39] demonstrated that the presence of metastatic epidermoid carcinoma to the lymph nodes, larger tumor size, and better prognosis has a significant correlation with increasing p53 and Ki-67 expression on immunohistochemical examination. Martinez et al. in [35] reported that there was an increase in the expression of p53 gene immunohistochemistry in KSSM and dysplastic lesions in cigarette addicts of as much as 74%–78%. Figure 4 shows the expression of P53 in the OSCC case in several parts of the cell.

If p53 is not expressed on epithelial cells, then p63 and p73 are highly expressed on epithelial cells, which are the sites of carcinoma development. This indicates that p73 and p63 have tissue specificity for solid epithelial tumors compared to p53 [40]. Furthermore, p73 has a role in malignant changes in squamous cell carcinoma. Functional loss of p73 was associated with squamous cell carcinoma due to the change from initiation to malignancy in the model mouse, followed by near-total sporadic loss of p73 mRNA and protein expression, with only a slight decrease in p63 protein expression [41]. Activation of p73 is sufficient to induce apoptosis in cancer cells and even causes tumor regression in mice, regardless of the status of p53. This is interesting, considering that p53 is mutated in 50% of cancerous cells and functionally inactive in up to 90% of them, making it difficult to reactivate as a therapeutic target [42]. Genetic changes in p53 lead to immortalization and predisposition of cells transformed into a neoplasm. This mortality is related to the maintenance of telomere length by telomerase. hTERT is a key component of telomerase whose activity is suppressed by p53. The hTERT protein expressed by OSCC strain cells is associated with the mutant state p53. The presence of hTERT protein expression in normal oral mucosal tissue is thought to be caused by keratinocyte and hematopoietic cell infiltration [43, 44].



**Figure 4.** Representative photomicrographs of [A] immunohistochemistry performed on a consecutive section show p53 expression extending to the suprabasal cell layers (x200). [B] Oral squamous cell carcinoma, grade III, original magnification 400x. [C] a representative section at 100 magnification demonstrating high (3+) p73 expression [D] high c-jun marker expression. [E] high c-fos marker expression. In approximately 45% of epithelial cells (40), nuclear hTERT staining was moderate. [Image courtesy of Haraguchi et al. in [44], Venkatesh et al. in [48]. Matsha et al. in [49], Wang et al. [50].

C-Fos is a proto-oncogene that acts as a transcriptional activator and binds to c-Jun to form a complex called AP-1 [45]. As a member of the AP-1 family, c-Fos plays an important role in proliferative and differentiation signal transduction cells and is an important gene in carcinogenesis regulation, suppressing tumor suppressor genes, resulting in invasive cancer growth. Cells from C-Fos cause loss of polarity and transition of mesenchymal epithelium,



resulting in invasive growth and metastasis of cells from breast epithelium [46]. Based on Seousa et al. In [47], we found that both c-Jun and c-Fos were found to be expressed in normal oral mucosa and OSCC. In normal mucosa, c-Jun immunoreactivity was found in the cytoplasm of the upper basal layers, whereas in OSCC, c-Jun was found in the cell nuclei. C-Fos expression was found in cell nuclei in both normal mucosa and OSCC, but it differed depending on the cell layer in normal mucosa and the differentiation of OSCC. Below is an immunohistochemical examination of tumor cell marker expression and protooncogenes p53, p63, p73, c-jun, c-fos, and hTERT.

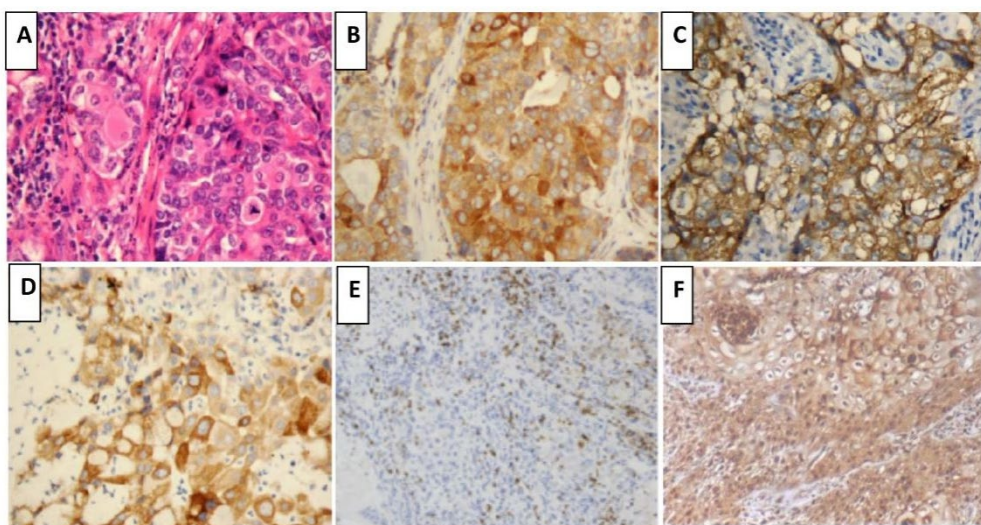
## **6 Immunohistochemical examination with angiogenic growth factors VEGF, MMP1, MMP-9, EGFR, TGF- $\alpha$**

Treatment and prognosis are determined by general tumor growth, including angiogenesis, which is the process of forming new blood vessels and distributing nutrients and oxygen to growing tumor cells. The angiogenesis process is stimulated by vascular endothelial growth factor (VEGF). VEGF expression was investigated in various types of cancer to determine the function of VEGF in the development and invasion of bladder cancer. The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase that strongly influences carcinogenesis. Some tumors, such as head and neck tumors, colon, lung, breast, bladder, and prostate cancers, show overexpression. VEGF-enhancing from tumor cells triggers the proliferation of endothelial cells. Several studies have shown that EGFR stimulation induces VEGF expression and is involved in VEGF control [51].

Normal cells require exogenous growth signals to stimulate proliferation. This growth signal is sent from cell surface receptors, which then gradually activates intracellular signaling pathways that produce proliferation. According to Wee and Wang in [52] study, they explained that during carcinogenesis, there was an increase in growth factor receptors with their ligands, which resulted in autocrine stimulation without exogenous factors. Increased expression of the Epidermal Growth Factor (EGFR) receptor, Transforming Growth Factor (TGF- $\alpha$ ), and several signaling proteins Intracellular cells play an important role in tumor development. TGF- $\alpha$  is a polypeptide with a molecular weight of 6 to 20 kilodaltons that has strong mitogenic activity. TGF- belongs to the growth factor family, which also includes epidermal growth factor (EGF). TGF- $\alpha$  and EGF share a core sequence of six characteristically spaced cysteines with three intracellular disulfide bonds, allowing them to interact with the same cellular receptor, the epidermal growth factor receptor (EGFR). Kannan et al. [53] discovered an increase in immunoreactive TGF- $\alpha$  in PVL and OSCC. TGF-  $\alpha$  immunoreactivity was seen in the cytoplasm of the basal and suprabasal layers of normal oral mucosa and was not significantly different across the oral cavity. TGF- $\alpha$  immunoreactivity was found in both basal and suprabasal cells in OSCC, and the COD was higher than in normal oral mucosa, indicating an increased level of TGF- $\alpha$  expression in these lesions.

The EGFR molecule activates the MAPK pathway, phosphatidylinositol-3-kinase (PI3K), AKT, mammalian Target of Rapamycin (mTOR), Janus Kinase (Jak), Signal Transducer and Activator of Transcription (STAT), and Protein Kinase C (PKC), inducing cancer cell proliferation and survival, invasion, metastases, and angiogenesis. Hepatocyte Growth Factor/c-Met is a receptor kinase that shows sufficient expression in OSCC, thereby increasing the motility or spread of cancer cells via paracrine pathways that increase MMP-1 and MMP-9 expression. Another growth signal is CycD1, which is a proto-oncogene encoding regulator positive for the G1 phase of the cell cycle that initiates DNA synthesis. Ras-oncogene is a proto-oncogene involved in the regulation of cell growth and transmitting mitogen signals from the cell surface into the cell nucleus.

Jin et al. in [54] designed their study to investigate the relationship between VEGF, EGFR and MMP-9 in non-small cell lung carcinomas (NSCLC), Expression of VEGF, EGFR and MMP-9 was related to pathology grading, lymph node metastasis and clinical staging in NSCLC ( $P < 0.01$ ), while being independent of other clinicopathologic parameters. There was an obvious positive correlation between the expression of VEGF and EGFR, VEGF and MMP-9, and EGFR and MMP-9 in NSCLCs. While in Cai et al. in [55] study, investigated the levels of angiogenic factors and MMPs in tumor tissue and saliva of OSCC patients. They found that the levels of HGF, VEGF, PIGF, MMP-1, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13, and TIMP-2 were upregulated both in OSCC tissue and in the saliva of OSCC patients. Patient survival and cancer functional states in head and neck cancers indicate that they could be saliva-based non-invasive diagnostic/prognostic markers and therapeutic targets for OSCC. The immunohistochemical examination with angiogenic growth factors VEGF, MMP1, MMP-9, EGFR, and TGF- is shown in Figure 5.



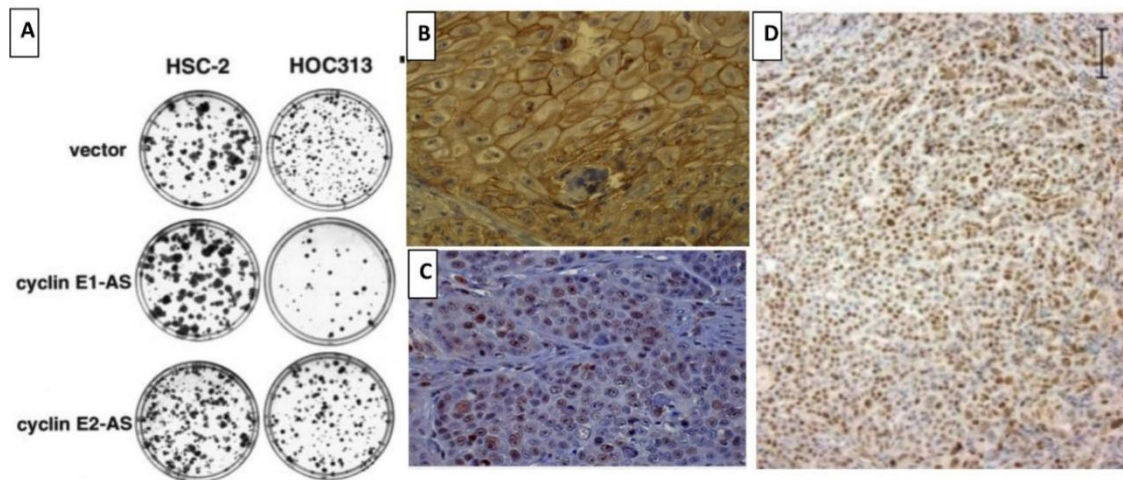
**Figure 5.** Representative photomicrographs of [A] Haematoxylin and eosin x 200 [B] VEGF in the cytoplasm [C] EGFR in cytoplasm and membranes [D] MMP-9 in cytoplasm; IHC x 200 [E] The stronger the expression of MMP-1 [F] High grade OSCC showing positive reaction for TGF- $\alpha$  [picture was taken from Abbas, et al. in [56], Fan, et al. in [57]. Jin et al. in [54].

## 7 Immunohistochemical analysis of Cyclins D (CDK-1) and E (CDK-2), Prb, c-erbB2, or HER2/neu as cell cycle regulators.

The cell cycle starts with the entry of cells from the G0 phase (quiet) to the G1 phase due to stimulation by growth factors. Early G1, Cdk4 and/or 6 phases are activated by Cyclin D (CycD) [58, 59]. The Cdk4/6 complex with CycD will initiate phosphorylation of the retinoblastoma protein family (pRb) during early G1. The Rb protein is key to the regulation of the G1 phase and affects the phosphorylation of Cdk. In the early phase of G1, pRb is found in hypophosphorylated concentrations and binds tightly to the transcription factor E2F, thereby suppressing its action. However, during the G1 phase, pRb becomes hyperphosphorylated at the Cdk attachment site, and therefore transcription occurs [60]. This condition causes the cells to be able to pass the restriction point at the end of the G1 phase and enter the S phase. This phosphorylation activity is a continuous series of processes initiated by Cdk4 and Cdk6 associated with CycD [61]. The subsequent phosphorylation effect on CycE causes the relationship between pRb and deacetylated histones, which should maintain the cohesiveness of the chromatin structure, to be disrupted or chromatin re-formation occurs [62]. This causes the DNA structure to loosen and the transcription factors that were originally bound to pRb are released, and the transcription of the E2F-responsive gene required in the cell cycle progression to the S phase becomes active. This cycle is the path of Rb [63].

Each stage in the cell cycle is tightly controlled by cell cycle regulators, namely cyclins. The main types of cyclins in the cell cycle are Cyc D, E, A, and B. Cyclins are expressed periodically, so that the concentration of cyclin varies in each phase of the cycle cell. Cdk2 and DNA synthesis are activated by CycE [64]. Different from other cyclins, CycD is not expressed periodically but is always synthesized during growth factor stimulation. The major Cdk proteins in the cell cycle are Cdk4, 6, 2, and 1 [65]. Different from other cyclins, CycD is not expressed periodically but is always synthesized during growth factor stimulation. The major Cdk proteins in the cell cycle are Cdk4, 6, 2, and 1. Based on Joh et al. in [67], in OSCC and PMD, age, gender, and site had no statistically significant correlation with cyclin D1 expression. The cyclin D1 score did not correlate with the histopathological diagnosis of OSCC. Cyclin D1 was not expressed in 60% of control and 30% of PMD cases, but it was expressed in 100% of OSCC cases, despite the fact that cyclin D1 score did not show a statistically significant association with disease prognosis among OSCC patients. While in Yamada et al. in [68] about cyclin E in OSCC, their study indicates that HSC-2, Regulation of Cell Motility via high and low affinity autocrine motility factor (AMF) receptor in human oral squamous carcinoma cells, cells lost proper growth control specifically mediated by cyclin E and suggests that deregulation of its downstream pathway may contribute to tumorigenesis of oral SCC. Meng et al. in [69] found that there was an overexpression and significantly correlated with the poor prognosis in OC patients. In addition, c-erbB-2 overexpression was associated with the following clinicopathological features of OC patients: occurrence risk, gender, lymph node metastasis, and differentiation grade. Figure 6:

Immunohistochemical analysis of Cyclins D (CDK-1) and E (CDK-2), Prb, c-erbB2, or HER2/neu as cell cycle regulators.



**Figure 6.** Representative photos of [A] Inhibition of cyclin E activity Photographs of plates Cyclin E activity is inhibited in colony formation assays of HSC-2 and HOC313 cell lines, [B]. Membranous c-erbB2 or HER2/neu Staining in oral Squamous Cell Carcinoma ( $\times 400$ ), [C] high intensity of pRb in OSCC. Almost all of the neoplastic nuclei have high intensity immunostaining for pRb protein, [D] strong (+++) expression levels of cyclin D1 in oral SCC tissues . [Photos by Sardari et al., [70], Suppavhad et al., [71], and Ohnishi et al., [72].

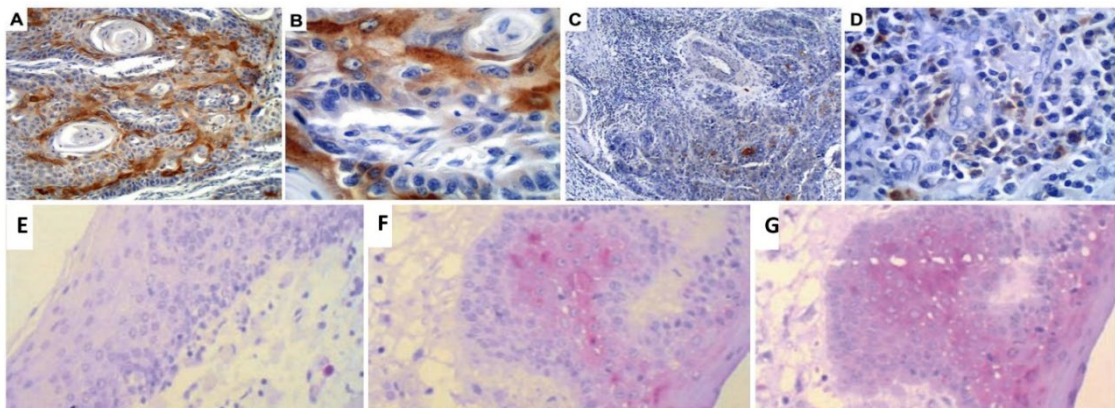
## 8 Immunohistochemical examination with inflammation marking enzymes COX-1 and COX-2

Arachidonic acid metabolism is thought to play a significant role, especially when it comes to carcinogens [73]. This metabolic pathway is related to the formation of prostanoids. Prostanoids are included in the eicosanoids subclass that undergoes conversion into prostaglandins, thromboxane and prostacyclin [74]. Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid into prostaglandins, which was first identified 20 years ago. Some studies state the presence of prostanoids in the pathogenesis of OSCC. In this study, in vitro, showed that growth factor, tumor promoters (tumor promoters) and oncogenes induce prostanoid synthesis [75]. Other research through in vivo studies showed that the intensity of COX-2 staining increased significantly from well to poorly differentiated OSCC. The intensity of staining differed significantly between moderately and poorly differentiated SCC. In poorly differentiated SCC, the percentage of positive tumor cells was higher than in well and moderately differentiated OSCC. According to Thomas et al. [76], the administration of chemoradiation therapy combined with COX-2 should be evaluated to improve therapy response.

COX-1 works as housekeeping on most normal networks. The COX-2 enzyme is an inducible form. The PG form derived from COX-1 activity facilitates various physiological processes, while COX-2 is highly induced by various inflammatory processes, growth factors, and other tumor promoters [77]. Through fluorescence microscopy examination and histofluorescence staining technique, the images of Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) were



located on the endoplasmic reticulum and nuclear membrane. COX-2 concentrations were higher in the nuclear membrane [78]. With the development of medical science, they found 3 families of cyclooxygenase (COX), namely COX-1, COX-2, and the latest, Cyclooxygenase-3, which have the same enzymatic activity but have different functions and expression patterns. COX-1 and COX-2 are products of two different genes. COX-1 in humans is located on chromosome 9 and COX-2 on chromosome 1. physiology of this tissue. Although COX-2 is present in very small amounts in normal tissues and although its active time is short, it is an intermediate-early response gene that increases the expression of 20-fold growth factors, tumor promoters and oncogenic mutations, while COX-3 is mostly found in the cerebral cortex and heart [79]. The study about families of Cyclooxygenase (COX) in OSCC by Pannone et al. [80] compared the expression of COX-1 and COX-2. They found that higher levels of COX-2 expression were associated with poor disease-free survival but not with overall survival and higher tumor stage and grade. Meanwhile, COX-1 plays a role in oral carcinogenesis and could be regarded as a potential therapeutic target by chemopreventive drugs. Moreover, COX-2 expression might be addressed as a new prognostic tool in the clinical management of OSCC. Fig 7 shows the immunohistochemical examination with inflammation marking enzymes COX-1 and COX-2.

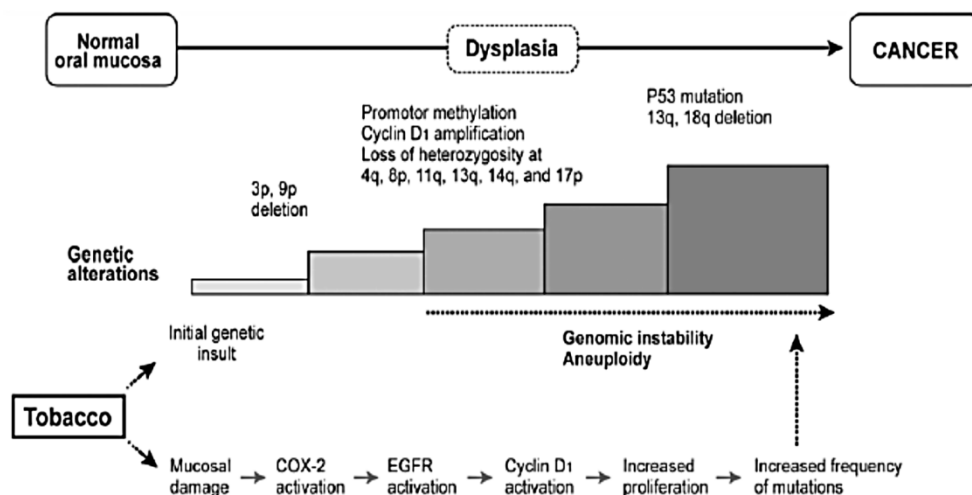


**Figure 7.** Representative photomicrographs of differential immunoexpression of COX-2 and COX-1 in OSCC (A) Strong and granular immunoexpression in the cytoplasm (IHC staining 400); (B) Overexpression in low grade OSCC (IHC staining 200). (C) Subexpression in high-grade OSCC (IHC staining 200); (D) Weak expression in neoplastic cells, but immunostaining in inflammatory cells (IHC staining 400). COX-1 expression was determined in specimens of head and neck squamous cell carcinomas and in normal oropharyngeal mucosa by immunohistochemistry (paraffin sections; fast red as chromogen): (E) Normal oropharyngeal epithelial cells show no COX-1 immunoreactivity (F). The  $\alpha$ -COX-1 antibody detects COX-1 in tumour cells although it is incubated with the COX-2-recombinant-control-protein. However, the complex of  $\alpha$ -COX-1/COX-1-recombinantcontrol-protein labelled weakly tumour cells (G). [Image courtesy of Thomas et al. in [76], Erovcic et al. in [80].

## 9 Immunohistochemical examination with loss of heterozygosity (Chromosome loci 3p, 8p, 9p, 4q, 11q, 13q, 17p).

Carcinogenesis is a multi-step process that begins with the loss of the arms of chromosomes 3p and 9p. This condition occurs in normal tissue changes to dysplasia. It is followed by the loss of several other chromosomal arms, such as 8p, 13q, and 17p. Loss of tumor suppressor genes is suspected to occur at sites where p16 loss occurs at 9p and the gene p53 is missing at 17p. Loss of heterozygosity (LOH) at 9p is seen in 72% of lesions and appears at the p16 site encoding cell cycle protein that functions to inhibit Cdk so that excessive cell proliferation may occur. The disruption process on the chromosomal arm can continue and will cause changes in hyperplasia, dysplasia, and ultimately carcinoma in situ.

Indirect clinical evidence demonstrated the involvement of smoking in the development of squamous cell carcinoma. The proportion of smokers who suffer from carcinoma is two to three times greater than non-smokers. The risk of recurrence in smokers is as much as two to six times as compared to people who quit smoking. (Figure 8) shows a model genetic development in a study of gene changes in OSCC in the oral cavity and head.



**Figure 8.** OSCC multistep genetic development model. Change normal epithelium by multiple genetic changes causing dysplasia and invasive carcinoma. Accumulated changes genetic factors that occur in carcinogenesis include activation of EGFR, alteration of p53 and p16 tumor suppressor genes and expression of excessive cyclin D1. [Source: Choi, et al. in [81]]

Yoo et al. [82] conducted research on the sequential molecular changes and genetic progression models of laryngeal SCC. This research proves that on the change from normal oral mucosa to dysplasia, some gene changes occur, including missing chromosomes 3p and 9p, then at an advanced stage, follows changes in loss of heterozygosity (LOH) on some chromosomes, namely 4q, 8p, 11q, 14q, and 17p. At the stage of change from dysplasia to cancer, p53 gene mutation and loss of chromosomes 13q and 18q are important. During this process, tobacco is also thought to be responsible for mucosal damage and to initiate genetic disorders. Mucosal damage activates

cyclooxygenase-2 (COX-2), epidermal growth factor receptor (EGFR) and cyclin D1, which increases cell proliferation and mutation frequency.

Mao, *et al.* in [82] in their trial studied 84 leukoplakia samples from 37 patients for two microsatellite markers located at chromosomes 9p21 and 3p14. Their results showed that 51% of patients (19/37) in their study group showed LOH on either or both loci. In order to validate their previous 2000 model, this prospective cohort included 296 patients with a histological diagnosis of primary mild or moderate dysplasia. Patients were classified into high- or low-risk profiles. According to the findings of their prospective study, high-risk lesions with 3p and/or 9p LOH had a 22.6-fold increase in risk when compared to low-risk lesions with 3p and 9p retention. When another two markers (loci on 4q and 17p) were added to the analysis, the risk prediction was improved even more, with 5-year progression rates of 3.1%, 16.3%, and 63.1% for low risk (9p retention), intermediate risk (9p LOH or 9p LOH with either 17p LOH or 4q LOH but not both), and high risk (9p LOH) [83].

They also stated that 9p21 was more important than 3p14 as a predictor of progression of oral premalignant epithelial conditions in this prospective study, and that LOH on 3p may be a passenger alteration rather than a driving force for progression. Graveland *et al.* examined exfoliated cells and biopsied tissue from 43 leukoplakia patients (6 of whom developed oral cancer) to examine LOH profiles at chromosomes 3p, 9p, 11q, and 17p, as well as immunohistochemical staining of biopsied tissue for p53 and TP53 mutation analysis. LOH was discovered to be present in 51% of the 9p cases. These findings also confirmed the significance of mutated TP53 and LOH at 9p in the biopsy as individual and combined markers [84].

## **10 Immunohistochemical examination with a marker protein formed by the body's cells as a result of infection with HPV**

Although not conclusively proven, oncogene (tumor-producing) viruses play an important role in the development of cancer. Viruses can integrate with host genetics and impair the body's ability to control infected cell growth and proliferation. Virus oncogenes can cause infected cells to live, facilitating the transformation to malignancy. Several studies have found that retroviruses, adenoviruses, herpes simplex virus (HSV), Epstein-Baar virus (EBV), and human papillomavirus (HPV) are all cancer-causing agents (HPV). Human papillomavirus (HPV) is a key player in the development of oral cavity tumorigenesis. Only HPV has been linked to cancers of the oral cavity, pharyngeal tonsils, larynx, esophagus, uterine cervix, vulva, and penis. HPV is a type of DNA virus. There are 90 different types of HPV, but only 30 of them have been linked to anogenital cancer, with the highest risks being HPV 16 and HPV 18, while HPV 6 and HPV 1 have a low risk of causing cancer. Subtypes 16, 18, 31, and 33 of the human papillomavirus can cause dysplasia and SCC.

The virus papillomavirus subtype 16 has a significant relationship with OSCC, whereas HPV 18 plays a minor role in the development of carcinoma. Herpes simplex virus type 2 was once thought to cause uterine cancer and cavities in the mouth, but it has now been proven that HSV does not play a role in cancer formation. Yete et al. [85] conducted research on human papillomavirus (HR-HPV) types 16 and 18. The potential use of de-intensified therapy and prophylactic prevention in patients with HPV-positive oral cancer is highlighted. Meanwhile, Dalakoti et al. [86] published a study on the prevalence of HPV in oral squamous cell carcinoma in the south-west of India.

## 11 Conclusion

Immunohistochemical examination can help reveal the pathogenesis of OSCC. The examination was carried out on biopsy material of cancerous lesions in the mucosal tissue. The pathogenesis of OSCC that can be detected through immunohistochemical examination includes the involvement of carcinogenesis proteins (with EGFR and TGF antibodies), apoptotic-specific proteins in OSCC (survivin antibodies), cell cycle regulatory factor proteins (cyclin D antibodies), and proliferation proteins (Ki-67 protein with antibodies). detector, namely Mib-1), as well as a typical oncogene virus that triggers OSCC (papilloma virus subtype 16). Disclosure of the pathogenesis of OSCC by immunohistochemical examination opens up opportunities for intervention in the progression of OSCC.

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