Chapter One
Introduction

Oral cancer (OC) which is the 6th most common cancer worldwide, continues to be the most prevalent cancer related to consumption of tobacco, alcohol and other carcinogenic products (Nelson, 2005). While the incidence of this cancer remains high in the Sudan, particularly, among men due to the habit of (Toombak use Tobacco Specific Nitrose amine (TSN) in rich tobacco) (Ahmed and Mahgoob, 2007). Oral cancer accounts for almost 3% of cancer cases in the world (Silva, et al. 2011). Oral cancer is an important cause of morbidity and mortality worldwide with an incidence rate that varies widely by geographic location. Even within one geographic location, the incidence varies among groups categorized by age, sex, site or habit (Preeti, et al. 2010). There have been reviews which assess the evidence of the relationship between human papillomaviruses (HPV) and oral cancer the evidence is increasing (Scully, 2002; Mayne, et al. 2006). Infection with the HPV is an important co-factor in the development of oral carcinomas. Initially, HPV infects undifferentiated proliferative basal cells, which are capable of dividing there is also strong molecular evidence supporting the role of HPV (particularly HPV-16) in the pathogenesis of oral cancer (Howley, et al. 2001). HPV-16 accounted for the majority of oropharyngeal cases (87%), compared to oral cavity cases (68%); while HPV-18 was rare in oropharyngeal cases (3%), but higher in oral cavity cases (34%). Gillison and Shah (2001) found an HPV prevalence of 25% in over 250 cases of

oropharyngeal tumours (90% HPV-16). In recent study by Ahmed and Eltoom (2010) on detection of HPV Types 16 and 18, among Sudanese patients with oral squamous cell carcinoma (OSCC), they found that evidence supporting causal association between HPV infection and OSCC in Sudan.

The diagnostic workup can be divided into history, physical examination, cytology, biopsy, and imaging studies. The most common type of oral cancer is squamous cell carcinoma (SCC). SCC originates in abnormal mucosa as either leukoplakia, erythroplakia or speckled leukoplakia in clinical assessment. Tissue biopsy is the 'gold standard' required to diagnose oral cancer. The tissue specimen taken then undergoes histopathological processing and examination to determine the pathological diagnosis.

Immunohistochemical staining has revealed the presence of HPV capsid antigens in HPV-infected cells. Capsid antigens, however, has rarely been detected in high-grade neoplasia or invasive cancer, probably because such tissue contains limited numbers of highly differentiated squamous epithelial cells (Syrjanen, et al. 1990).

The expression of cytokeratin 19 (CK19) in transformed oral lesions has been regarded as an early feature in the premalignant and malignant transformation and invasive potential of OSCC. Furthermore, the altered CK19 was observed at obnormal intraepithelial levels in normal mucosa from OSCC (Bosch, et al. 1989; Ogden, et al.1993). Therefore, overexpression of $p16^{INK4A}$, as detected by immunohistochemistry, has shown to be a useful

adjunct to cytology in oral cancer screening (Sahebali, et al. 2004), a reliable marker of human papillomavirus-induced oral high-grade squamous dysplasia (Cunningham, et al. 2006).

Polymerase Chain Reaction (PCR): PCR is the most sensitive HPV detection method, where even a single gene copy of HPV is possible to detect. PCR detection of HPV is highly sensitive and specific, and can supplement the detection of clinical manifestations of virus-associated oral lesions (Scully and Carrozzo, 2008). In clinical studies, PCR techniques using either consensus primers or type-specific primers have been employed to elucidate the importance of the various HPV genotypes (Camisa, et al. 1998).

Prevention: The majority of OSCC is related to tobacco and alcohol use and hence the best way to prevent OSCC would be to educate the general population about the risk. Furthermore, identifying individuals with inherited dysfunction in their DNA repair systems, cell cycle control and apoptotic pathways rendering them sensitive to tobacco induced carcinogenesis would have a great impact on prevention as well as early detection (Sturgis, et al. 2004). In addition prevention of HPV induced OSCC would include changes in sexual behaviour since each alteration of sexual partner increases the risk for HPV infection (Smith, et al. 2004).

The recognition and management of precancers therefore constitutes a vital oral cancer control measure. Oral precancer is distinguished into precancerous lesions and conditions. Precancerous lesion is a morphologically altered tissue in which

cancer is more likely to occur than its apparently normal counterpart (Fali, et al. 1993).

The treatment of oral cancers mainly consists of surgical treatment, radiotherapy, chemotherapy and immunotherapy as auxiliary treatments. Precancerous lesions, such as patches of thin leukoplakia are observed and do not require biopsy or treatment. Cessation of etiologic factors such as tobacco and alcohol will help prevent progression to carcinoma (Million, et al. 1994). The prognosis of OSCC patients remains largely unsatisfactory, due to loco-regional recurrence. The 5-year survival rate is less than 50%, and the prognosis of advanced cases has not improved much over the past three decades. Lack of biomarkers for early detection and risk assessment is clearly reflected by the fact that more than 50% of all OSCC patients have advanced disease at the time of diagnosis (Neville and Day, 2002; Bettendorf, et al. 2004)

1.1- **Rational:**

The strong association between oral cancers and HPV, is mainly found in certain ages of people who practice oral sex, in many countries including United States (Smith, et al. 2004). In Sudan, there are considerable numbers of oral cancers, most of them are attributed to the use of toombak, but there is no evidence highlighted the relationship between these cancers and HPV or low association between HPV and OSCC among Sudanese patients (Ibrahim, et al.1998). A recent study in Sudan by Ahmed and Eltoom (2010) they found that evidence supporting causal association

between HPV-16 and HPV-18 infection. Therefore, this study will investigate the role of other genotyping or all high risk of HPV, among Sudanese patients with oral neoplastic and non-neoplastic lesions.

1.2- Research objectives:

1.2.1. General objectives:

To identify the difference subtypes of High Risk human papillomaviruses (HR-HPV), among Patients in Khartoum State with oral lesions.

1.2.2. Specific objective:

1- To identify HR-HPV subtypes by using, Immunohistochemical and molecular methods.

2- To compare between Immunohistochemical and molecular methods.

3- To co-relate between demographic data and HPV.

Chapter Two

Review of Literature

2-1. Scientific background:

2.1.1- Infections and inflammatory changes:

2.1.1.1- Bacterial Infections:-

Focal oral infections can be defined as infections occurring in different locations of the human body and which are caused by microorganisms (or their products) inhabiting the oral cavity. Three different mechanisms by which oral bacteria may cause non-oral diseases have been described: first metastatic infection caused by translocation of bacteria, second metastatic injury related to microbial toxins, and third metastatic inflammation due to immune injury (Thoden Van Velzen, et al.1984). Although this concept remains controversial, it has been given more attention by the dental and medical communities. This is largely due to improvements in methods of sampling, cultivation and identification of bacteria that revealed the presence of microorganisms well known to be oral colonizers in a variety of infected non-oral sites (Destefano, et al. 1993). Bacterial Sinusitis and Tonsillitis: Acute and chronic sinus infections are most commonly caused by bacterial organisms, including Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, other streptococcal strains, and anaerobic bacteria (brook, 2005).

The spread of periapical infections into tissues surrounding the oral cavity may give rise to chronic maxillary sinusitis and fascial plane infections. These infections may lead to breakdown of the fascia surrounding the muscles by an acute inflammatory process and the lytic enzymes of the microorganisms. The breakdown of the fascia allows the purulent exudate to spread throughout the immediate region. Swelling of

the posterior floor of the mouth, such as Ludwig's angina, is life threatening because it can compromise the airway (Van Velzen, et al. 1984).

The oral cavity contains some of the most varied and vast flora in the entire human body and is the main entrance for 2 systems vital to human function and physiology, the gastrointestinal and respiratory systems. Several diseases involve these 2 systems and manifest in the oral cavity. In addition, a specific pathologic condition, such as periodontitis (inflammation of the periodontal attachment of the teeth and the alveolar bone), may be present in the oral cavity. Periodontal diseases can be grouped into two major categories, gingivitis and periodontitis (Marsh & Martin, 1992; Slots & Rams, 1992). Gingivitis is defined as an inflammation of the gums surrounding the teeth. It is associated with plaque accumulation around the gingival margin. In a healthy gingival crevice, the total number of microorganisms is small and the microbiota is dominated by facultative gram-positive bacteria (Slots & Rams, 1992).

Bacteria play a definite role in periodontal disease, but specific agents that produce the disease have not been identified. As with caries, it appears that periodontitis is induced by an association of several microorganisms. Many other oral cavity bacteria probably play a role in this process, however. Calculus at the gingival margin contributes to the periodontal disease process. Associated with calculus is Actinomyces naeslundii; this bacterium multiplies substantially with the increasing age of the plaque. An organism not frequently considered in regard to periodontal periodontal disease patients and low or absent in those who do not have the disease; Periodontitis involves the destruction of the connective tissue attachment and the adjacent alveolar bone (Slots & Rams, 1992). It is a severe form of gingivitis with gingival crevice forming a periodontal pocket due to the apical migration of the junctional epithelium along the root surface. It can cause progressive loss of the

alveolar bone around the teeth and lead to the loosening and subsequent loss of teeth (Slots & Rams, 1992). Several processes including plaque accumulation, release of bacterial substances, and host inflammatory response are involved in the induction and progression of periodontal tissue destruction (Genco, 1992).

Oral mucosa changes typically include buccal cobble stoning, orofacial granulomatosis, lip swelling, angular cheilitis, hyperplastic changes (eg. gingival hyperplasia), deep linear sulcal ulcerations, aphthae, cheilitis, stomatitis, changes of mucosa coloration, taste disorders and lichen planus. Those changes may be caused by malnutrition, but also may have their cause in incorrect function of the autoimmune system, or have genetic origin (Jasyk & Paradowska, 2008).

Bacillary Angiomatosis: (BA) is a systemic disease caused by B henselae and Bartonella quintana. Histologic examination of BA lesions shows ectatic vessels lined by plump, cuboidal endothelial cells that may show marked pleomorphism and mitotic activity and, therefore, may be confused with malignancy. The presence of neutrophils and fibrin adjacent to blood vessels may aid in the diagnosis (Turgut, et al. 2004).

The chancre sore of primary syphilis can involve oral mucosa. Patients with secondary syphilis may have mucosal erosions on the tongue, lips, and oral mucosa. Gummatous lesions of tertiary syphilis may involve mucous membranes. Oral lesions in primary and secondary syphilis are nonspecific and characterized by squamous hyperplasia and a plasma cell infiltrate that extends deep into the submucosa (Zawar, et al. 2005).

Inflammation and Irritation; Many of the lesions that are classified as inflammatory or irritative are denture-related, including buccal irritation along the occlusal plane (frictional hyperkeratosis), denture "sore spot" and denture "sore mouth". Frictional

hyperkeratosis is caused by chronic friction against an oral mucosal surface, resulting in a hyperkeratotic white lesion, analogous to a callus on the skin. The lesion is a protective response to low-grade, long-term trauma. When the friction occurs along the buccal mucosa in the line where maxillary and mandibular teeth contact each other, it can produce a white line called linea alba. If the physician is clinically confident of a traumatic cause for the lesion, no biopsy is required. Removal of the cause of irritation usually resolves the problem. If the cause is uncertain, the lesion should be treated as idiopathic leukoplakia, and biopsy should be obtained. Any of these factors could cause small, painful ulcers, characterized by an overlying, grayish necrotic membrane and surrounded by an inflammatory halo. Treatment is aimed at correcting the underlying cause. After the cause is removed, the 6 ulcer usually heals quickly. Biopsy should be obtained if the lesion does not heal in two to three weeks following elimination of the suspected cause (Donald & James, 1991).

Hyperkeratosis (focal keratosis) is an increased thickness of the keratin layer of stratified squamous epithelium with no microscopic evidence of atypical epithelial cells. Clinically, hyperkeratotic lesions appear as white, rough, non-painful patches that do not rub off. They are often secondary to chronic irritation, such as biting or tobacco use. Hyperkeratotic lesions on oral mucosal surfaces that are normally keratinized, such as dorsum of the tongue, hard palate, and attached gingiva, sometimes represent a physiologic response (callus) to chronic irritation. Remember, however, that dysplasia, carcinoma in situ, and squamous cell carcinoma can occur on any oral mucosal surface (Michael, et al. 2010).

2.1.1.2- Fungal Diseases:

Acute atrophic candidiasis is usually associated with a burning sensation in the mouth or on the tongue. Diagnosis may be difficult but should be considered in the differential diagnosis of a sore tongue especially in a frail older patient with dentures

who has received antibiotic therapy or who is on inhaled steroids. A swab from the tongue/buccal mucosa may help diagnosis. Candida albicans infections of the oral cavity are primarily observed in young children. Oral candidiasis (thrush) is detected less frequently in adults and then usually as a chronic disease. Thrush consists of discrete or confluent white patches, composed of hyphae and yeast cells, on the mucous membranes of the oral cavity. Oral candidiasis responds to topical application of nystatin to the lesion. The nystatin is either painted on the affected area or administeredas nystatin lozenges. Gentian violet (1%) will also control this disease (Silverman, et al. 1984).

Chronic hyperplastic candidiasis characteristically occurs on the buccal mucosa or lateral border of the tongue as speckled or homogenous white lesions. There is an association with smoking (Silverman, et al. 1985) and complete resolution appears to be dependent on cessation of smoking. This condition can progress to severe dysplasia or malignancy and is sometimes referred to as candidal leukoplakia. Candida spp are not always isolated from lesions of oral leukoplakia and it has been suggested that the finding of Candida spp in these premalignant lesions is a complicating factor rather than a causative one (Dreizen, 1984).

Chronic atrophic candidiasis also known as “denture stomatitis” is characterised by localised chronic erythema of tissues covered by dentures. Lesions usually occur on the palate and upper jaw but may also affect mandibular tissue. Diagnosis requires removal of dentures and careful inspection; swabs may be taken for confirmation. Median rhomboid glossitis is a chronic symmetrical area on the tongue anterior to the circumvallate papillae. It is made up of atrophic filiform papillae. Biopsy of this area usually yields candida in over 85% of cases. It tends to be associated with smoking and the use of inhaled steroids (Budzt-Jorgenson, 1990).

Angular cheilitis is an erythematous fissuring at one or both corners of the mouth, and is usually associated with an intraoral candidal infection. Other organisms implicated are staphylococci and streptococci. In the case of staphylococci the reservoir is usually the anterior region of the nostrils and spread to the angles of the mouth has been confirmed by phage typing (Kanbe, 1991).

2.1.1.3- Viral Diseases:

A variety of viral and virally related diseases show oral cavity manifestations. Viruses are associated not only with inflammatory or nonspecific systemic conditions but also with neoplasias.

Acute tonsillitis and oropharyngitis are frequently caused by viruses: Viral causes of tonsillitis include Epstein-Barr virus (EBV), herpes simplex virus (HSV), parainfluenza virus, measles virus, coxsackievirus, and adenovirus. Paramyxovirus (mumps virus) infects the parotid and submandibular gland, resulting in salivary gland enlargement. Detailed pathologic changes of these infections will not be discussed (Smith, et al. 1999; Dohil, et al. 2006).

Human immuno-difiency virus (HIV): Infection with HIV (an RNA Lentivirus) can manifest in several ways in the head and neck area. Almost 70% of HIV-infected patients will have lesions involving the oral cavity (Chidzonga, 2003; Reznik, 2006). Sinusitis, mucosal ulceration, candidiasis, salivary gland enlargement, lymphadenopathy, chronic sinusitis, and many other entities may be seen. It is believed that the incidence of oral lesions associated with HIV has been decreasing with antiviral therapy (Campanini, et al. 2005; Hillel, et al. 2004).

Cutaneous Lesions: Several skin lesions are seen more frequently in HIV+ patients. These include KS, BA, seborrheic dermatitis, psoriasis,viral infections, pruritic papular eruptions, eosinophilic folliculitis, and cutaneous carcinomas (squamous cell

carcinoma and basal cell carcinoma) (Rigopoulos, et al. 2004; Wilkins, et al. 2006; Raju, et al. 2005).

Herpes simplex viruses (HSV): These can cause significant pathology in the head and neck area. Primary herpesvirus infections are often followed by the development of latent viral infections in a variety of organ systems. HSV, cytomegalovirus (CMV), varicella zoster virus (VZV), EBV, and human herpesvirus (HHV)-6, HHV-7, and HHV-8 can have manifestations in the oral cavity (Huber, 2003; Stoopler, 2005; Arduino & Porter, 2006).

Cytomegalovirus (CMV): infection may result in oral cavity manifestations, including extensive mucosal ulceration, especially in the oropharynx and the sinonasal tract. Patients have sore throat, odynophagia, and dysphagia. (Syrjanen, et al. 1999; Leimola, et al. 1995; Chan, et al. 2002). Oftentimes, the symptoms overlap with those seen in infectious mononucleosis. Clinically significant infections are seen more frequently in immunosuppressed patients. Retinitis is also a known result of CMV infection (Wiegand & Young, 2006; Scholz, et al. 2003; See & Rao 2002).

Epstein - Barr virus (EBV): It has been implicated in several disease processes. The majority of adults have serologic evidence of exposure to the virus. Infections are often asymptomatic in young children. In adolescents and young adults, infection often results in the development of infectious mononucleosis. In infectious mononucleosis, EBV infects B cells resulting in B-cell activation and proliferation (Ebell, 2004; Cohen, 2003)

HPV Infection: It has been implicated in a variety of papillomatous and malignant squamous proliferations, including those seen in the oral cavity area such as sinonasal papillomas and squamous cell carcinoma (Almadori, et al. 2002; Baez, et al. 2004). Benign HPV types induce lesions characterized by hyperplasia, parakeratosis and

papillomatosis. The differences in these features vary between HPV types. High risk HPV types can potentially induce lesions with intraepithelial neoplasia characterized by disorganized architecture of the epithelia, abnormal mitotic figures and nuclear atypia. These lesions are graded depending on how much of the epithelia that are affected. In addition, in HPV infected cells halos appear around the nucleus, a phenomenon that known as koilocytosis (Baez, et al. 2004).

2.1.2- Benign Neoplastic Oral Lesion:

Benign tumors are typically better defined or circumscribed and have a slower growth rate, measured in months and years, than malignant neoplasms (Michael, et al. 2010).

The most common causes of benign oral mucosal swellings are cysts arising in the minor salivary glands of the lower lip (mucoceles) and inflammatory overgrowths resulting in fibroepithelial polyps or hyperplastic tissue related to ill-fitting dentures.

Squamous cell papillomas are relatively common benign neoplasms that occur on the oral mucosa. Viral warts are 'papilloma-like' lesions that occur in response to a virus, usually the HPV.

Pyogenic granuloma is a pedunculated hemorrhagic nodule that occurs most frequently on the gingiva and that has a strong tendency to recur after simple excision. It is an erythematous, non-painful, smooth or lobulated mass that often bleeds easily when touched. Oral pyogenic granulomas most often develop on the gingiva, but less common locations include the lip, tongue, and buccal mucosa (Sills, et al. 1996; Kroumpouzos and Cohen, 2001).

*Squamous papilloma***.** The most common benign epithelial neoplasm of oral epithelium, squamous papilloma lesions may be found anywhere in the mouth with a predilection for the ventral tongue and frenum area, palate, and mucosal surface of the lips (Silverman, et al. 2002). The typical treatment is surgical excision. All lesions

resembling a squamous papilloma are recommended for excision at the base (1-mm margin) to the depth of the submucosa.4 Removal should also be considered the cure. Recurrence or appearance of new lesions suggests the possibility of retransmission of a condyloma acuminatum or a carcinoma (Marx & Stern, 2003).

Pleomorphic adenoma, also known as mixed tumor, is the most common tumor of salivary gland origin. The parotid gland is the most common location. The posterior lateral quadrant of the hard palate is the most common location for tumors of minor salivary glands, but it may be found in any mucosal region that contains salivary glands. Pleomorphic adenoma has clinical features similar to many other benign tumors arising from salivary glands and mesenchymal tissue. Complete surgical removal is the treatment. Very rarely carcinoma arises in a preexisting pleomorphic adenoma (Michael, et al. 2010).

Focal epithelial hyperplasia: This lesion is typically located in the labial, buccal, and lingual mucosa. Focal epithelial hyperplasia lesions usually resemble the normal mucosal color but may occasionally appear white and papillary (Silverman, et al. 2002).

Fibroma: It is a focus of hyperplastic fibrous connective tissue representing a reactive response to local irritation or masticatory trauma. Fibromas occur in approximately 1.2 percent of adults. The most common location is along the occlusal line of the buccal mucosa, an area subject to masticatory trauma, although other locations, such as the tongue, labial mucosa, and gingiva, are possible (Nevile, et al. 2002).

Verruca vulgaris: Also known as the common wart, verruca vulgaris the most prevalent HPV skin lesion, but it can also be found in the oral cavity. This lesion is usually associated with HPV 2 and 4. In the mouth, verruca vulgaris is found most commonly on the keratinized surfaces of the gingiva and palate. Verruca vulgaris

lesions are contagious, and it is thought that some oral lesions occur following autoinoculation. These lesions are typically found in children but can be seen in any age group (Silverman, et al. 2002).

Haemangiomas are relatively common developmental lesions of the oral mucosa and may cause cosmetic problems if affecting the lips. Their appearance can be of a network of red capillaries in the mucosa or a nodular lesion filled with blood, which may blanch on pressure. Haemangiomas may bleed if traumatized but this is uncommon. Hemangioma, characterized by a proliferation of blood vessels, is often congenital. Hemangiomas in the oral cavity are flat or raised, with a deep red or bluish-red color. They are seldom well circumscribed. The most common sites are the lips, tongue, buccal mucosa and palate (Scully, 2008).

Lifestyle changes have also been tied to the rise in HPV-associated intraoral infection. An increase in the number of people engaging in premarital sex, multiple sexual partners, and oral sex over the past few decades has likely contributed to increased rates of oral HPV infection (Boyle, et al. 2003). In line with this trend, a recent U.S. study has reported an association between oral sex and open-mouthed kissing and the development of oral HPV infection (D'Souza, et al. 2009). A case-control study demonstrated that a high number (≥26) of lifetime vaginal-sex partners and six or more lifetime oral-sex partners were associated with an increased risk of HNSCC (D'Souza, et al. 2007).

2.3- Oral Cancer:

Cancer develops, as Weinberg (1998) simply puts it, from ‘one renegade cell’ and when ‘cells grow out of control’. ‘Tumour’ is the non-specific term for a lump or swelling, and tumours are characterised as either ‘benign’ or ‘malignant’ (the characteristic of the latter being its invasiveness into surrounding normal tissue, while

the former are considered ‘non-cancerous’ and do not spread (‘metastasise’) to distant parts of the body (National Cancer Institute, 2007a). The term ‘metastasis’ characterises the highest degree of tumour malignancy and usually is the cause of death in cancer patients (National Cancer Institute, 2007a). The term cancer is used to encompass a widely diverse range of diseases – almost all cells in the body can give rise to a particular form of cancer, but multiple forms of cancer can also develop from each cell type. Thus, cancer is considered primarily by its anatomical location or site, and secondly by its cell type (Percy, et al. 1990).

Pathologists grade tumours by grade of differentiation. Poorly differentiated tumours, where the tissues and cells do not have the same appearance as the tissue of origin, are generally highly malignant. Tumour stage refers to the stage of tumour development at the time of presentation and clinical diagnosis: generally, the higher the stage, the worse the prognosis. Several factors contribute to tumour stage including: tumour size, extent of invasiveness, positive lymph node spread, distant metastasis (Barnes, et al. 2005).

Oral cancer itself has many definitions, based mainly on the debate around the coding of anatomical sites to include in the classification of the disease (Moore, et al. 2000). However, the debate perhaps lies deeper than with epidemiologists and pathologists coding of sites. Malignant neoplasms are more likely to be painful and cause ulceration of the overlying epithelium than benign lesions (Michael, et al. 2010).

Anatomists themselves seem unable to fully agree on a definition of the ‘oral cavity’, ‘the mouth’, and the ‘oropharynx’. Squamous cell carcinoma (SCC) is by far the most common epithelial malignancy in the oral cavity. SCCs and their variants constitute over 90% of oral malignancies. The continuum of SCC progresses from individual

epithelial cell changes (atypia), to a generalized disturbance of the epithelium (dysplasia), then to carcinoma in situ, and finally to invasive SCC (Hong, et al. 1993). The terms “precancer,” “precursor lesions,” “premalignant,” “intraepithelial neoplasia,” and “potentially malignant” have been used in the international literature to broadly describe clinical presentations that may have the potential to become cancer. They all convey the a priori assumption that there is uniformity in how individual patients and tissues behave. The terminology ought to reflect our best understanding of carcinogenesis in the oral mucosa, and to aspire to engender consistency in use. The latest WHO monograph on oral tumors uses the term “epithelial precursor lesions” (Barnes, 2005). Oral epithelial dysplasia is subdivided into three prognostically significant categories: mild (grade I), moderate (grade II), and severe (grade III). Over 90% of OSCCs are preceded by preexisting potentially malignant lesions (Noonan & Kabani, 2005). Although some primary tumors can be treated, many patients will develop second primary tumors, suggesting multifocal tumor development. According to an International Agency for Research on Cancer report (GLOBOCAN, 2008) (IARC, 2008), oral cancer is the tenth most common cancer for men and fourteenth for both sexes in the world in terms of number of cases, accounting for approximately 480,000 new cases and mortality of 275,000 per year. In Sweden, the incidence of oral cancer is 869 and the mortality is 256 per year. The incidence of oral cancer in Sudan is 1547 and the mortality is 929 per year. Oral cancer in India is among the five most frequent cancers after cervical and breast cancer; among the oral tumors, 90% are SCC, which arises in the mucosal lining. This high incidence of oral cancers in India is due to the widespread habits of tobacco chewing and smoking (Das & Nagpal, 2002). The broad ethnic and climatic diversity of Sudan makes it in many ways a microcosm of Africa. Sudan is experiencing a

burgeoning cancer epidemic that carries many challenges characteristic of developing countries. These include a high incidence of advanced, difficult-to-treat disease at presentation, and a high cancer burden related to infectious diseases. To address this problem, Sudan has instituted a comprehensive national cancer control program focused on prevention, early detection, improved treatment, and palliative care (Hamad, 2006).

2.3.1- Epidemiology of oral cancer:

Global mortality of oral cancer is relatively high in both developed and developing countries with approximately 207,000 deaths (compared to the 390,000 newly diagnosed cases) estimated in 2000 (Ferlay, et al. 2001) and approximately 40,000 new cases and almost 8,000 deaths from OSCC are estimated to occur in the United States in 2012 (Siegel, et al. 2012). In Sudan the incidence of oral cancer is 1547 and the mortality is 929 per year and the incidence rate was 13.5%, the mortality was 13% and the males females ratio was 1.3:1 (GLOBOCAN, 2008). Over the last decade, mortality from oral and pharyngeal cancer has been declining in most European countries, but it had been increasing substantially in Hungary, Slovakia and a few other countries of central Europe (Martina, et al. 2011). Tongue cancer was the most common cancer of oral cavity among Iranian patients and similar epidemiologic and clnicopathological characteristics of the disease were found in patients (Ebrahim, et al. 2011). According to an International Agency for Research on Cancer report (GLOBOCAN, 2008), oral cancer is the tenth most common cancer for men and fourteenth for both sexes in the world in terms of number of cases, accounting for approximately 480,000 new cases and mortality of

275,000 per year. In Sweden, the incidence of oral cancer is 869 and the mortality is 256 per year, also Sweden and other western countries, the prevalence of oral cancer is higher for men than women, probably because men consume more alcohol and tobacco than do women (Nylander, et al. 1995). In Sweden, the incidence of oral cancer is 869 and the mortality is 256 per year. In India is 118,424 and the mortality is 89,411 per year (GLOBOCAN, 2008). While in the United States, cancers of the oral cavity account for nearly 2.3% of cancers and also have a relatively low five-year survival rate (Casto, et al. 2009).

In Sudan; OC is the fifth most common cancer type with about 920 cases per year, comprising 9% of the cases reported annually in Africa (Ferlay, et al. 2005). An gepidemic of oral cancer predicted by 2020 in India they found that it is predicted that if pan masala habit is increasing at the present rate, there will be an epidemic of oral squamous sarcinoma by 2020 in India (Mathew, 2009). The use of Toombak has been stated to play a major role in the etiology of oral cancer in the Sudan and is suspected to be associated with neoplasm of salivary glands (Elbeshir, et al. 1989 ; Idris, et al. 1994 ; Idris et al. 1995). In the Sudan, Toombak, was introduced approximately 400 years ago. It is always processed into a loose moist form and its use is widespread in the country (Idris, et al. 1992). This is a very popular material in the Sudanese community (Idris, et al. 1998). In addition Hilmi, et al. (2008) in the international congress Khartoum 2008, suggested that smokeless tobacco might have an

antiviral effect that may inhibit HPV infections. The study done by Ahmed and Mahgoob (2007); Ginawi, et al. (2012), they found that 86.6% of oral cancers among Sudanese patients occurring in males with mean age of 48 years. In another recent study, oral HR-HPV infections showed a bimodal age pattern, with peak prevalence at 30–34 year of age and another peak at 60–64 year of age (Gillison, et al. 2012). HPV16 is the most common genotype in oral lesions, in which HPV16 and 18 are the most prevalent HPV genotypes. Another recent study by Brew, et al. (2012), reported that, HPV 16 in 6 cases (75.0%), co \infections HPV 16/18 and 16/33 in one case each (12.5%). Many studies found a relatively high frequency of oral malignant neoplasms, particularly squamous cell carcinomas in men from Northern Sudan and of the Gaalein tribe, who lives in northern Sudan. Eastern side has shown relatively high occurrence of OSCCs with the absence of HPV infection, but no study from there has explored the possible causes (Ahmed and Mahgoob, 2007; Idris, et al. 1995; Ahmed and Eltoom, 2010). In very recent study reported by Fernández, et al. (2013), they found that A high frequency of HPV (67%) infection in the oral and genital mucosas, suggesting that patient's habits could contribute to the infection; the presence of HPV in the oral mucosa may act as reservoir for new HPV infections. Another study by Ahmed and Mahgoob (2007); Ginawi, et al. (2012) that men accounted for over 74%. Oral cancer in Sudan is lower among females. This is because toombak use (synergistic factor to HPV) is uncommon among females, as it is considered as a social stigma in the Sudan. Markopoulos (2012), the OSCCs is the most common malignant epithelial neoplasm affecting the oral cavity. A similar study by Choi and Myers (2008) reported that more than 90% of all oral neoplasms are OSCC. According to Globocan report 2008, the incidence of oral cancer in Sudan was high compared with other part of the world e.g. in Egypt 6.4%, Ethiopia 11.6%, Chad

6.7%, Europe 5.5%. In the United States it is 4.5%, in India 19.9% and in Australia it is 4.2% (Globocan, 2008). However, the exact mechanism behind this variation is not well defined but it might be due to geographic variation and various etiological risk factors. There were over 5500 new cases of oral cancer (lip, mouth, tongue and oropharynx) diagnosed in the UK in 2004, thus outnumbering cervical cancer. Although this represents only a small percentage (2%) of all UK cancers, the number of new cases is increasing with a trend for a greater proportion of younger people and women being affected (Cancer Research UK, 2010). Oral cancer is more common in men than women and the majority of cases in the UK occur in people aged 50 years or over. The survival rate for patients with oral cancer depends on the staging of the disease at presentation and the site of the lesion. The prognosis is poor for those with advanced disease, who present at a late stage; those surviving have to cope with the functional and cosmetic consequences of radical treatment. Cancer of the lip has the best outcome with 90% of patients surviving 5 years and most being completely cured. (Cancer Research UK, 2010). Rogers, et al. (2010) looked at the reasons patients delayed in presenting symptoms of oral cancer to a medical specialist. The majority of patients felt the initial symptoms (non-healing ulcers, sore mouth or a persistent lump) were trivial and believed they would heal of their own accord. This study highlights the need for greater awareness of the symptoms of oral cancer to encourage patients to present their symptoms earlier, and improve disease outcomes (Rogers, et al. 2010).

2.3.2- Etiology of oral cancer:

Two major etiological factors in oral cavity SCC are social habits of tobacco use and alcohol consumption (Proia, et al. 2006). No single factor causes oral cancer; it is a

combination of extrinsic and intrinsic factors over time and is dependent on each person's unique response to both known and unknown risk factors. The most modifiable risk factors are tobacco and alcohol use, followed by betel quid and poor ppdiet. Other risk factors that can be ascertained in a dental office include age, a previous history of oral cancer or precancer, and Immunosuppression. Awareness of human papilloma virus (HPV) status and exposure to ultraviolet light may not be easily identifiable on a patient health history. In very recent study by Ahmed, 2013, reported that, Toombak use and infection with high risk Human Papilloma Virus (HPV) were extensively investigated and linked to the aetiology of oral cancer.

2.3.2.1- Tobacco:

Tobacco use is widely considered the most important and dominant risk factor for oral cancer (Boyle, et al. 1990; Mucci and Adami, 2002; Mayne, et al. 2006). Tobacco cessation is the most valuable form of primary prevention. The highest percentage of smokers in Canada is in the 20-24 year age group (Health Canada, 2008). Within oral cancer in the western world, tobacco is most associated with cancers of the floor of the mouth. The risk of oral cancer and premalignant lesions increases both with the amount and the length of time tobacco has been used (Reichart, 2001). A meta-analysis found current smokers had a relative risk of oral cancer of 3.43 versus non-smokers (Gandini, 2008). Patients who continue to smoke after an oral cancer diagnosis are at a greater risk for a second oral cancer. Fortunately, the risk for premalignant lesions, oral cancer and second oral cancers decreases once a smoker has quit and continues to decrease. After 10 years of not smoking a former smokers risk of oral cancer is on par with that of a never smoker (La Vecchia, 1999). There has been an increase in the use of smokeless tobacco in young men. Tobacco companies are advertising it as a safer alternative to smoking yet more than 30 carcinogens are

found in smokeless tobacco (Boffetta, 2008). As with smoked tobacco there is dose-response relationship with smokeless tobacco. In the United States, the use of smokeless tobacco more than doubles the risk of oral cancer according to a recent meta-analysis. In India smokeless tobacco products accounts for almost 50% of oral cancer (Boffetta, 2008). Smokeless tobacco has been associated with a greater risk of premalignant lesions such as erythroplakia and leukoplakia (lARC, 2007). One study found that almost 15% of smokeless tobacco users had an intraorallesion at the site where they placed their tobacco (Kaugars, 1992). Betel quid is a popular product chewed primarily by people of South Asian descent. Betel quid is made up of a betelleat areca nut, lime (calcium hydroxide) and flavouring agents. The lime, used to hold the ingredients together, is an irritant and will erode the tissue in the oral cavity. While betel quid can be chewed without tobacco it is more common to have tobacco included. Betel quid without tobacco is also known to be a carcinogen. As with tobacco, risk is associated with dose and duration of use. In India, where betel quid chewing is widespread, the most common site for oral cancer is the buccal mucosa (Hashibe, 2002 ; Berthiller, 2009).

The use of Toombak has been stated to play a major role in the etiology of oral cancer in the Sudan and is suspected to be associated with neoplasm of salivary glands (Elbeshir, et al. 1989; Idris, et al. 1994; Idris, et al. 1995). In the Sudan, Toombak, was introduced approximately 400 years ago. It is always processed into a loose moist form and its use is widespread in the country. Tobacco used for manufacture of the Toombak is of the species Nicotiana rustica and the fermented ground powder is mixed with an aqueous solution of sodium bicarbonate (Idris, et al. 1992).

2.3.2.2 – Alcohol

The heavy use of alcohol is also a major risk factor for oral cancer. While there is no reported minimal threshold for alcohol risk (Bagnardi, 2001), men who drank more than 21 units per week and women who drank more than 14 units of alcohol per week were found to have an increased risk of oral cancer (Johnson, 1997 ; Cancer Research UK, 2008). One unit of alcohol is equal to 1 - 4 ounce glass of wine, 1- 8 ounce relationship, risk of oral cancer increases both with the duration of alcohol consumption and with the amount consumed. There are some geographical differences found with risk as the processing of alcohol can vary by country. In the past, the risk from alcohol alone has been difficult to study as subjects who drank but did not smoke were not common. More recent studies have identified alcohol as a significant risk factor on its own. An increase in oral cancer in people in their 40s in the United Kingdom is believed to be due to alcohol use (Cancer Research UK, 2009). Unlike tobacco, the risk for oral cancer in former heavy drinkers can persist for more than 20 years after a person quits drinking. How alcohol acts as a carcinogen is not fully known. Some theories include acetylaldehyde, a known carcinogen and metabolite of alcohol, and ethanol causing DNA damage; nutritional deficiencies associated with heavy drinking; the carcinogenic effect of chemicals other than ethanol in alcoholic beverages; the induction of microsomal enzymes that enhance the metabolic activation of other carcinogens; or the capacity of alcohol to enhance the penetration of carcinogens (tobacco) in the tissue (Rehm, 2007). Together tobacco and alcohol have a synergistic effect and account for approximately 75% of oral cancers. Heavy smoking and drinking in men can increase the risk of oral and oropharyngeal cancer by 38 times, and in women a 100 fold increased risk has been found (Blot, 1988). The greatest risk for developing an oral cancer or premalignant lesions is for patients who both smoke and drink, with decreasing risk for non-drinking smokers

and non-smoking drinkers, respectively. Similarly non-smoking, heavy drinkers had twice the increased risk of head and neck cancer and attributed for 7% of the cancer cases. Alcohol use is very rarely documented by the oral health professional, again preventing an opportunity to educate patients about their oral cancer risk and the use of alcohol in moderation (Rehm, 2007).

2.3.2.3 – Age:

The majority of oral cancers are diagnosed in the 7th decade of life. This is a result of the accumulated lifelong exposure to carcinogens and damage to DNA. The risk for men 40 – 59 years of age is 11 times that of men under the age of 40. This risk increases to 21 times once men reach the age of 60. Women over the age of 40 years have 5 times the risk and over 60 have 12 times the risk of women under the age of 40 (Statistical Research and Applications Branch National Cancer Institute, 2009). While oral SCC is predominantly a disease of the elderly, there appears to be an increase in the incidence of oral cancer in young adults under 40 years of age (Mendez, 1985; Annertz, 2002). The gender difference observed in older oral cancer patients is not evident in this group and females appear to be more prominent in the young cancer sufferers (Popovtzer, 2004). For younger oral cancer patients, under the age of 40 years, there appears to be 2 variations. One group is similar to older oral cancer patients with similar risk habits and disease progression (Liewellyn, 2004). The second group develops oral cancer without a known etiological cause and their cancer seems to progress quicker than most oral cancers (schantz, 1988; Annertz, 2002). This group is often composed of young females who present at a more advanced stage possible due to diagnostic delays attributed to their lack of known risk factors. This group may be at an increased risk due to inherited risk factors or altered immunity. Oral health professionals must also be aware of the increase in oral cancer in younger

patients so that oral cancer screening is not limited to older patients or that clinical lesions are not ignored due to the younger age of a patient (Annertz, 2002).

2.3.2.4- Diet:

A person with a diet low in fruits and vegetables is at an increased risk for oral cancer. The combination of poor nutrition with tobacco and alcohol use is believed to account for 85% of oral cancers (Shah, 2003). A multiplicative effect was also found for oropharyngeal cancer between a diet low in fruits and vegetables and heavy smoking or alcohol use (Tavini, 2001). A diet which includes the recommended daily allowance of fruit and vegetables can reduce the risk of oral cancer by half. Citrus fruits, orange and yellow vegetables have been found to be particularly protective. In a meta-analysis of 16 studies regarding nutrition and oral cancer, a 49% reduced risk for each portion of fruit eaten and a 50% reduced risk for vegetables consumed was discovered. Citrus fruits had a greater effect than total fruits consumed and were also found to reduce the risk of oropharyngeal cancer, particularly when consumed for a long duration. The protective effect of fruits, citrus fruits and vegetables against oral cancer and second oral cancers, has been supported by other research (Chainani, 2002; Freedman, 2008). The role of diet in oral cancer is important information for oral health professionals both for educating their patients about prevention but also in the follow-up of patients with oral premalignant lesions (OPL) and cancer (Pavia, 2006).

2.3.2.4- Human papilloma virus associated cancers:

Human papilloma virus (HPV) has a wide disease spectrum affecting the cutaneous and mucosal areas of the body, ranging from benign common warts to invasive carcinoma. HPV infections have been reported in a number of body sites, including the anogenital tract, urethra, skin, larynx, tracheobronchial mucosa, nasal cavity,

paranasal sinus, and oral cavity. Oral HPV infection may be associated with different diseases of oral cavities (Campisi, et al. 2007).

There have been several narrative reviews which assess the evidence of the relationship between human papillomaviruses (HPV) and oral cancer (Scully, 2002; and Mayne, et al. 2006), and the evidence is increasing. More than 100 different strains of HPV have been identified to date. HPV can potentially be transmitted through sexual contact including oral sex. Infection with HPV most commonly is associated with genital warts or papillomas which are benign epithelial lesions. However, the causal association of mucosal human papillomaviruses (HPV) with cervical cancer in particular, but also with cancers of the male and female genitals, and the anus, is becoming well established – with up to 10 strains of HPV being implicated (Walboomers, et al. 1999; Bosch and De Sanjose, 2003). There is also strong molecular evidence supporting the role of HPV (particularly HPV-16) in the pathogenesis of oral cancer. Kreimer et al. (2005) undertook a systematic review and pooled analyses from over 5,000 head and neck cancer specimens from 60 studies. They found an overall HPV prevalence of around 25%, which was higher in oropharyngeal cancers (36%) than in oral cavity cancer (24%). HPV-16 accounted for the majority of oropharyngeal cases (87%), compared to oral cavity cases (68%); while HPV-18 was rare in oropharyngeal cases (3%), but higher in oral cavity cases (34%). Gillison and co-workers (2000) from the Johns Hopkins School, Baltimore, in the first of a series of studies in this area, found an HPV prevalence of 25% in over 250 cases of oropharyngeal tumours (90% HPV-16). HPV infection is well established as an etiological agent responsible for a number of pathologies affecting the stratified epithelia of skin and anogenital sites. More recently, the infection by (mucosal) high-risk HPV types has also been found to be causally associated with squamous cell

carcinoma in the head and neck region (HNSCC), especially in the oropharynx (Kostareli, et al. 2012). HPV16 is the most common genotype in these tumours but HPV6 and HPV11 can also be found in a minority of these cancers, implying that these low-risk HPV types are not entirely benign in the head and neck region (Syrjanen, 2012). In the Johns Hopkins follow-up study investigating behavioural risk factors, oral HPV infection was found to be strongly associated with oropharyngeal cancer among subjects, independent of tobacco and alcohol use (D'Souza, et al. 2007). Additionally, in 2005, in the largest case-control study to date, an international multi-centre IARC coordinated study examined data on 1,670 cases and 1,732 control subjects and found an almost 3-fold increased risk for oral cavity cancer and an over 9-fold increased risk for oropharyngeal cancer (Herrero, et al. 2003). The interest in HPV in the aetiology of oral cancer is also associated with the finding that those tumours which are HPV positive are associated with a better prognosis. This was also shown in the Johns Hopkins series, with oropharyngeal cancer patients with HPV positive tumours having an almost 60% reduced mortality rate compared to those with HPV-negative tumours (Gillison and Shah, 2001; Gillison, 2004). Such findings have also led to growing interest in the possibility of HPV-vaccinations for oral cancer (D'Souza, et al. 2007).

Molecular structure and role of high and low risk HPV in OSCCs: Human Papilloma virus is about 55 nm in diameter. It has a single circular double stranded DNA molecule and belongs to the family papillomaviridae. Its genome is made up of 7,200 – 8,000 base pairs with a molecular weight of 5.2 × 106 D. On the basis of DNA base pair (bp) distribution, the viral DNA is divided into three parts: first a 4,000 bp region that responsible for viral DNA replication and cell transformation, second 3,000 bp region that encodes the structural proteins of the virus particles and last 1,000 bp non-

coding region (NCR) that contains the origin of viral DNA replication. (Zur, et al. 2000) suggested that the genomic HPV DNA has nine open-reading frame sequences (ORFS) –present on single strand of DNA and are divided into seven early (E) and two late-phase genes (L). The transcription of viral DNA is regulated by early phase gene, while the capsid proteins (involved in viral spread) are regulated by late phase gene. The early-phase gene (E) encodes the E1, E2, E5, E6, and E7 proteins. E1 and E2 gene products regulated the transcription and replication of viral proteins and E5 gene product transcribed from the episomal region of the viral DNA (Zur, 1996). The E6 and E7 oncoprotiens are usually under control of E1 and E2 inhibitory genes. These genes have the ability to de-stimulate the tumor suppressor function and regulate the functions of the p16, p53, and pRb proteins, resulting in apoptosis, DNA repair and cell cycle control and may finally lead to cellular immortalization. The non-coding, long control region (LCR) contains binding sites for the E1 and E2 gene products, located just upstream of the promoter sequence 97 (P 97) which controls the transcription of the E6 and E7 oncogenes (Boyer, et al. 1996).

*High and Low-Risk Type HPV Infections***:** HPV can be divided into so called low-risk, intermediate risk and high-risk HPV types, depending on if they are commonly isolated from benign or malignant cervical lesions (Lorincz, et al. 1992). HPV types that are found preferentially in cervical and other anogenital cancers have been designated as ‘high-risk’ types (zur Hausen, 1986). Conversely, those found primarily in genital warts and non-malignant lesions were labelled as ‘low-risk’ types. High-risk oncogenic viruses include HPV 16, 18, 31, 33, and 35 (de Villiers, 1989; Zur Hausen, 1991; Snijders, et al. 1994). HPV types 39, 45, 51, 52, 56, 57, 58, 59, and 61 may also be present in dysplastic and malignant lesions of other anogenital sites, although less frequently (Snijders, et al. 1994; Garlick and Taichman, 1991). HPV 16 is most

commonly found in squamous cell cancers of the cervix, whereas HPV18 predominates in adenocarcinomas of the cervix. (Schiffman, et al. 1993; Hurlin, et al. 1991). The molecular basis for the tissue specific distribution of these two high-risk HPV types is not clear. The low-risk HPV types include HPV6, 11, 13, 32, 34, 40, 42, 44, 53, 54, 55, and 63, which are usually associated with benign lesions such as cervical condylomas (zur Hausen,1977; de Villiers, 1989; Greer, et al. 1990). Functional differences between high-risk and low-risk HPV types correlate strongly with malignant conversion of infected cells. High-risk HPV types induce increased chromosomal abnormalities and aneuploidy in the cell and encode oncoproteins (E6, E7) that interact specifically with cellular proteins (p53, P16) engaged inregulation of cell growth and proliferation (Donaldson, et al. 1993; Farthing & Vousden, 1994) Because high- and low-risk HPV types primarily reflect studies of cervical neoplasia, it is likely that additional HPV types which are found less frequently in other malignant tissues may also share some of the properties with the high-risk HPV types. Sequence comparisons high-risk and low-risk HPV types have consistently revealed a single amino acid sequence difference at residue 21 (Asp 21 in HPV16 E7 protein corresponding to a glycine residue in low-risk HPV6 E7) (Sang & Barbosa, 1992). Consequently, given the level of intragenomic variability within an HPV type, single amino acid substitutions in low-risk HPV E7 proteins may readily promote features normally characteristic of highrisk HPV E7 oncoproteins (Sang & Barbosa, 1992).

2.3.3- Pathogenesis of oral cancer:

The most common type of oral cancer is epidermoid carcinoma (squamous cell carcinoma). Epidermoid carcinoma originates in abnormal mucosa as either leukoplakia, erythroplakia or speckled leukoplakia. Precursor lesions in the mucosal linings, by far the most knowledge on the pathogenesis of squamous cell carcinomas

has been obtained from oral cancers, probably because oral precursor lesions are the most frequently diagnosed of these cancers, and specimens are available for research. Oral leukoplakia, a white lesion in the mucosa of the oral cavity, is the most common precursor lesion of oral squamous cell carcinoma and its prevalence varies between 0.1% and 0.5% (Napier & Speight, 2008; Van der Waal, 2009). The reported proportion of oral leukoplakia that develops into cancer depends on various factors such as the study population, the definition of leukoplakia used and the length of observation time, but an annual transformation rate of 1-2% per year is a reasonable assumption (Napier & Speight, 2008; Van der Waal, 2009). Risk factors for progression are female gender, size of lesion and the presence and grade of dysplasia (Napier & Speight, 2008). Although criteria have been defined by the world Health organization, it is difficult to make an objective categorization of dysplasia owing to a high inter-observer and intra-observer variation in assessment. It is considered appropriate to actively treat leukoplakia, irrespective of the presence of dysplasia (van der Waal, 2009). unfortunately, there is no scientific evidence that any type of treatment is able to prevent squamous cellcarcinoma in these patients (Lodi, et al. 2006). Factors that may explain this are that the leukoplakia recurs despite removal or that cancer develops outside the visible lesion (Partridge, et al. 2000). Although chemoprevention may cause the regression of leukoplakia lesions, a decrease in cancer incidence has rarely been observed (Wrangle & Khuri, 2007). The problems with histological grading and treatment of leukoplakia have fuelled molecular studies to assess the risk for progression and to identify targets for treatment. Several studies have shown that the presence and number of cancer-associated genetic changes can be used to discriminate leukoplakias with a low risk from those with a high risk of malignant transformation (Schaaij-Visser, et al. 2010; Torres-Rendon, et al. 2009). To

avoid painful biopsies, saliva and exfoliated cells can be obtained as a source for biomarker-based risk assessment (Shpitzer, et al. 2009; Bremmer, et al. 2009). Oral leukoplakias are visible precursor lesions that are macroscopically recognized. However, there are several many precursor changes in the oral mucosa are not visible to the naked eye. In 1953, the term‘field cancerization’was proposed to explain the high propensity to develop local recurrences after treatment of OSCC and the high likelihood that multiple independent tumours will develop in the head and neck mucosa (Slaughter, et al. 1953). In 1996, the first genetic multi-step progression model for OSCC was postulated on the basis of the genetic characterization of morphological changes in the squamous epithelium (Califano, et al. 1996).

2.3.3.1- Molecular pathogenesis of OSCCs:

OSCC arises as a result of multiple molecular events that develop from the combined influences of an individual's genetic predisposition and exposure to environmental carcinogens (Califano, et al. 1996). Chronic exposure to carcinogens such as tobacco, alcohol, oncogenic viruses, and inflammation can damage individual genes as well as larger portions of the genetic material, including chromosomes. Accumulation of such genetic alterations can lead to the development of premalignant lesions and subsequent invasive carcinoma. These genetic alterations include activating mutations or amplification of oncogenes that promote cell survival and proliferation, as well as inactivation of tumor suppressor genes involved in the inhibition of cell proliferation. From these alterations of oncogenes and tumor suppressor genes, tumor cells acquire autonomous self-growth. Tumor cells thereby escape programmed cell death and replicate infinitely through the immortalization process by telomere lengthening. As OSCCs grow, invade, and metastasize, new blood vessel formation is critical. OSCCs, like most tumors, are able to create a blood supply by stimulating endothelial cell

proliferation and new blood vessel formation. During oral carcinogenesis, there is selective disruption of this process, such that pro-angiogenic factors predominate (Hanahan and Weinberg, 2000). This angiogenesis is an essential part of solid tumor formation. The subsequent progression of OSCC includes tissue invasion and metastasis. Invasion of adjacent normal tissue requires that cellular adhesion molecules, such as integrin and cadherins, are lost, to allow cancer cells to leave their primary site. OSCCs develop through a complex process, as mentioned above. Here, we discuss those process involving genetic alteration during multistep carcinogenesis, growth regulation, apoptosis, immortalization, angiogeneis, invasion, and metastasis (Hanahan and Weinberg, 2000).

2.3.4- Diagnosis of oral cancer:

A meta-analysis of reports from 1982-1997, examining the risk of HPV detection in normal oral mucosa, pre-cancerous oral tissue, and oral carcinoma, showed that the probability of HPV being detected in the oral mucosa increased with increasing degree of dysplasia (Miller and Johnstone, 2001). In a total of 4680 samples from 94 studies, these investigators reported that the pooled probability of detecting HPV in normal mucosa was 10% (95% confidence interval"CI" = 6.1-14.6). The likelihood of detecting HPV in benign leukoplakia was 22% (95%CI = 15.7-29.9), in intraepithelial neoplasia 26.2% (95%CI = 19.6-33.6), in verrucous carcinoma 29.5% (95%CI = 23.0-36.8), and in oral squamous cell carcinoma 46.5% (95%CI = 37.6-55.5). The pooled probability of detection of any high-risk HPV was 2.8 times more likely than for a low-risk subtype (0.24 [95%CI = 0.160.33] and 0.09 [95%CI = 0.06-0.13], respectively). HPV16 and 18 were detected in 30% of oral squamous cell carcinomas (OSCC), while other high-risk types were detected in less than 1% of these tumors. Although the likelihood of high-risk HPV being detected may be higher in samples of

squamous cell carcinoma, there was substantial heterogeneity in detection rates between studies. This may be attributed to several factors, including: variations in prevalence between geographic locations of the performed studies, and between head and neck subsites (Kreimer, et al. 2005a); multiple HPV detection methods (polymerase chain-reaction [PCR], in situ hybridization [ISH]. To reduce the variation in the literature of HPV DNA prevalence in the oral and oropharyngeal mucosa, one recommendation may be to design more sensitive PCR primers. There is increasing evidence that HPV infection may occur frequently in the normal oral mucosa (Lambropoulos, et al. 1997; Terai, et al. 1999; Kansky, et al. 2003), but this does not mean that the presence of the virus predicts progression to malignancy, since the majority of HPV infections may be transient rather than persistent.

Diagnosis of early lesions, according to Cawson, et al. (1995), depends on a high index of clinical acuity and a readiness to biopsy lesions on suspicion. Tissue biopsy is the ‘gold standard’ required to diagnose oral cancer. The tissue specimen taken then undergoes histopathological processing and examination to determine the pathological diagnosis. Until recently, histological examination of biopsies and ‘invasive’ imaging techniques (e.g. radiology) has been the only methods of diagnosis and assessment of tumour characteristics. Latterly, there has been increasing interest in optical spectrospcopy systems to provide real-time, non-invasive and in situ tissue diagnosis according to Swinson, et al. (2006). Clinical assessment, staging, and treatment are topics out with the scope of the thesis.

Biopsy： The correct diagnosis of a lesion is essential before initiating treatment, as different tumors behave differently and thus require different treatment plans. Biopsy is mandatory to confirm the histologic diagnosis. There are several forms of biopsy; excisional, incisional, punch core needle and fine needle aspiration biopsy (FNAB).

The majority of these techniques provide adequate tissue for histologic diagnosis. FNAB is inexpensive, safe, quick to perform, and especially useful for evaluation of neck node masses. Definitive treatment, however, should not be performed on findings of FNAB alone, the diagnosis still needs to be followed and confirmed by histologic examination. In the past, FNAB did not gain widespread acceptance for fear of needle tract implantation, however, this has now been found to be unsubstantiated (Barnes, 1996). The specimen is taken from the clinically most suspicious area, avoiding necrotic or grossly ulcerated areas, and more than one biopsy site may need to be chosen. In patients with enlarged cervical lymph nodes and an obvious primary in the oral cavity or oropharynx, the biopsy is always taken from the primary site and not from the lymph node. In such situations, fine needle aspiration cytology may be carried out to verify the involvement of the node. If no obvious primary site is found in patients presenting with neck nodes, fine needle aspiration of the lymph node can be performed to help establish the diagnosis. In patients for whom fine needle aspiration is non-diagnostic and SCC is strongly suspected, excisional lymph node biopsy is a last resort, as subsequent curative therapy may be compromised by this procedure. Patients with SCC of the oral cavity or oropharynx have a risk of multiple primary tumors in the pharynx or larynx, as well as in the tracheobronchial region and esophagus so routine panendoscopy is often performed to evaluate these sites (Barnes, 1996).

Cytological methods: Scraping cytological methods using to detect and assess presence and degree of premalignant and malignant oral cancer, after the cells have been collected, fixed and stained, the morphology of surface epithelial cells is observed under a microscope (Langlois, et al. 1993). Diagnosis of early lesions,

according to Cawson, et al. (1995), depends on a high index of clinical acuity and a readiness to biopsy lesions on suspicion. The technique is simple, non-aggressive, relatively painless and tolerated well by patients. It can be used for diagnosis of predictive methodology and identification of recurrent OSCC (Driemel, et al. 2007). Fine needle aspiration cytology (FNAC) is a method that is used to collect cells from the affected site. FNAC is an excellent method for identifying intraoral lesions, although it is limited to early diagnoses (Chua,et al. 2010; Chahwala, et al. 2009; Brennan, et al. 2010). The Oral brush test, which is also known as a brush biopsy, is used to collect samples from mucosal epithelial cells. A brush is used to scrape and collect epithelial cells from different histological layers. The samples are collected are subjected to fixation on a glass slide. Then, the various cytological, immunohistochemical and molecular characteristics of the cells are analyzed to determine the severity of the lesion (Fedele, 2009; Mehrotra, et al. 2008). This cytological test is highly sensitive for detecting dysplastic changes in high-risk mucosal lesions, but it has limited use in the detection of low-risk lesions (Fedele, 2009; Mehrotra, et al. 2008). In addition, toluidine blue is a dye that stains nucleic acids and can detect differences in the DNA ploidy of cells to assess the possible presence of oncogenic transformation.

Although light-based tools are useful, visual and histological diagnoses often fail. While most of the reports agree that they are acceptable methods for detecting carcinomas, they still have only limited value in the prediction of early mucosal lesions. These tests should be used as accessories to clinical examinations in the evaluations of high-grade oral lesions. The cytological study of oral cavity cells is simple and rapid, non-aggressive and relatively painless: it is thus well accepted by

patients and suitable for routine application in population screening programmes, for early analysis of suspect lesions, and for pre-and post-treatment monitoring of confirmed malignant lesions (Mehrotra, et al. 2008). For questionable cases, biochemical or molecular patient diagnoses may provide more accurate information in future.

Cytomorphometry: Ogden, et al. 1997, suggested that quantitative techniques, based on the evaluation of parameters such as nuclear area (NA), cytoplasmic area (CA), and nucleus-to-cytoplasm area ratio (NA/CA), may increase the sensitivity of exfoliative cytology for early diagnosis of oral cancers, since these techniques are precise, objective and reproducible. Cowpe, et al. (1985) demonstrated that exfoliative cytology is capable of detecting malignant changes, through estimation of NA/CA using the planimeter method in Papanicolaou-stained smears. This study, published in 1985, concluded that 50 cells were sufficient to provide indication of malignant changes. Since then, a number of studies have been carried out using the technique described by these authors to evaluate the influence of diverse systemic and external factors on NA, CA and NA/CA. In these studies planimeters have been replaced by semiautomatic image analysis techniques, which are faster, more accurate and more reproducible (Cowpe, et al. 1991; Ogden et al. 1993). Found that tissues undergoing malignant transformation typically show a reduction in CA before the reduction in NA. They also suggested that samples of healthy mucosa from the same patient provide the best control. Ramaesh et al. (1998), used cytomorphometric techniques to assess nuclear diameter (ND) and cytoplasmic diameter (CD) in normal oral mucosa, in dysplastic lesions and in squamous cell carcinomas. They found that CD was highest in normal mucosa, lower in dysplastic lesions, and lowest in SCCs. By contrast, ND was lowest in normal mucosa, higher in dysplastic lesions, and highest

in SCCs. These studies suggested that reduced nuclear size and increased cytoplasm size are useful early indicators of malignant transformation, and thus exfoliative cytology is of value for monitoring clinically suspect lesions and for early detection of malignancy.

Chemiluminescence: is a method by which the oral mucosa is visually diagnosed using diffuse chemiluminescent blue/white light (wavelength 490–510 nm) after oral rinsing with a one percent acetic acid solution (Farah & McCullough, 2007). The appearance of blue staining of the normal oral mucosa by this examination and the appearance of brighter staining with a distinct margin indicates transformed mucosa (it reflects light differently due to a higher nuclear/cytoplasmic ratio). This method is effective; however, it sometimes fails to detect very early lesions (Fedele, 2009).

Tissue fluorescence imaging: is commonly used for the detection of precancerous lesions. During cellular transformation leading to the appearance of oral premalignant lesions, tissue hyperkeratinization increases, and the accumulation of increased chromatin material and metabolites and changes in the stromal collagen matrix and elastin occur. These alterations may be detected by measuring changes in the wavelength (tissue autofluorescence) of light that is emitted by the tissue. Normal oral mucosa emits a pale green autofluorescence, while abnormal lesions exhibit decreased autofluorescence and appear darker with respect to the surrounding healthy tissue. This method may not be effective if the lesion is very small (Fedele, 2009).

Tissue fluorescence spectroscopy: is another technique by which a small optical fiber is used to produce light at various wavelengths for tissue excitation, and then, the fluorescence spectra are detected by a spectrograph (Fedele, 2009).These emitted spectra are analyzed by proper software to reach a definitive conclusion.

2.3.4- Conventional Histopathology (benign & malignant):

If a soft tissue enlargement appears to be a tumor, the clinician must next determine if the enlargement is benign or malignant. Benign tumors are typically better defined or circumscribed and have a slower growth rate, measured in months and years, than malignant neoplasms. Malignant neoplasms are more likely to be painful and cause ulceration of the overlying epithelium than benign lesions (Michael, et al. 2010).

The histology of oral cancer is almost always squamous cell carcinoma (SCC) – accounting for over 90% of all invasive tumours at this site (Mayne, et al. 2006). The characteristic histopathological features of SCC include: invasion of underlying deeper tissues, varying degrees of squamous cell differentiation and cellular pleomorphism, increased nuclear staining, and tendancy to metastasis to regional lymph nodes (Johnson, et al. 2005).

Oral squamous cell cancer is graded histologically as: well; moderately; or poorly differentiated carcinoma. Well differentiated tumours contain orderly stratification and heavy keratinization in a ‘pear formation’; moderately differentiated tumours have prickle cells, some stratification, and less keratinisation; and poorly differentiated tumours are still recognisable as squamous cell carcinomas but manifest prominent nuclear pleomorphisms and atypical mitosis (Johnson, et al. 2005). The severity of dysplasia related to malignant potential cannot be objectively quantified and a significant proportion of dysplastic lesions either remain static or even regress. There remains a lack of studies which follow-up large series of dysplastic lesions and attempt to assess the association between histological features and whether malignant change is observed, (Cawson, et al. 1995).

The patterns of lymphatic spread – one of the primary routes of oral cancer spread – were reviewed by Johnson, et al. (2005). As oral cancers spread through the lymphatic system, lymph nodes in the submandibular region and deep cervical chain may be

palpable. Cancers of the tongue and floor of the mouth show a higher tendency to regional metastasis than cancers of the lower lip. It should be noted that cancers may show ipsilateral, contralateral or bilateral lymphatic spread (Johnson, et al. 2005). The presence of a lymphocytic response may have prognostic value, as does the manner of invasion (pushing or spreading) (Johnson, et al. 2005). Spread can occur by local infiltration, lymphatic drainage (to cervical nodes in the first instance) and late spread via the blood stream. In decades, (Wesley, et al. 1997; Mehrotra, et al. 2004). They are seen a dramatic switch from histopathological to molecular methods of disease diagnosis and exfoliative cytology has gained importance as a rapid and simple method for obtaining DNA samples. Changes occur at the molecular level before they are seen under the microscope and before clinical changes occur. Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys to reducing the mortality, morbidity and cost of treatment associated with OSCC.

2.3.5- Immunohistochemical (IHC) method:

Immunohistochemistry or IHC refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara, 2005). IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). IHC staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors (Nguyen,et al. 2010). IHC is an excellent detection technique and has the tremendous advantage of being able to show exactly where a given protein is located within the tissue examined. It is also an effective way to examine the tissues .This has made it a widely used technique in theneurosciences, enabling researchers to examine protein

expression within specific brain structures (Malley & Pinder,et al. 2006). The method of HPV detection in tumor tissue varies between laboratories and includes polymerase chain reaction (PCR), in situ hybridization (ISH) technique for HPV DNA and E6/E7 messenger RNA (mRNA), and immunohistochemistry (IHC) for p16 protein.

2.3.5.1- *p16$^{INK4a:}$*

p16, a cyclin-dependent kinase inhibitor and G1/S cell cycle phase checkpoint regulator and a thwarted surrogate marker of HPV E7–mediated functional inactivation of retinoblastoma gene protein (pRB), is consistently overexpressed in cervical high-grade squamous intraepithelial lesion, adenocarcinoma in situ, squamous cell carcinoma (SCC), and adenocarcinoma (Tringler, et al. 2004; Kong, et al. 2007; Samarawardana, et al. 2010).

Overexpression of p16^{INK4A} is considered to be strong and consistent in HPV-induced cancers (Reuschenbach, et al. 2008). p16 overexpression also shows high correlation with HPV E6/E7 mRNA expression, a marker of transcriptionally active HPV infection (Hoffman, et al.2010). In addition, a recent study has shown that the outcome of HPV−/p16+ cases is not significantly different from HPV+/p16+ cases and that this was better than HPV−/p16−, suggesting that p16 overexpression confers a more favorable outcome (Lewis, et al. 2010).

Expression of p16 has been shown to correlate with significantly improved outcomes in patients with oral squamous cell carcinomas (Lewis, et al. 2010; Harris, et al. 2011). Overexpression p16INK4A might specifically identify HPV infections that are biologically relevant in the carcinogenesis of oral squamous cell carcinomas and cervical carcinomas. However, it should be noted that expression of p16INK4A is not limited to HPV-positive tumors, and the use of this marker alone as an indicator of

biologically relevant HPV infections inevitably entails the risk of including some HPV negative p16INK4A positive results (Lassen, et al. 2009). Furthermore, the association between p16INK4A expression and HPV infection has been firmly established in oropharyngeal SCC (Arafa, et al. 2008 & Robinson, et al. 2010).

The tumour-suppressor gene p16 mapped on 9p21 and the protein gene product $p16^{INK4A}$ play an important role in the regulatory mechanism known as the pRb pathway (Todd, 2002). pRb phosphorylation helps cell cycle progression, while the process is closely controlled by a variety of regulating enzymes, including $p16^{INK4A}$ as an inhibitor of phosphorylation, where pRb limits the expression of $p16^{INK4A}$ expression by a negative feedback process (Muirhead, 2006; Ruas, 1998; Nagpal & Das, 2003). Low expression of $p16^{INK4A}$ with consequent loss of function has been demonstrated in most OSCC, (Yuen, 2002; Ai, 2003; Rocco, 2001) but paradoxically $p16^{INK4A}$ overexpression has also been detected in 15–50% of OSCC (Muirhead, 2006; Ai, 2003; Weinberger, 2004; Karsai, 2007)

Overexpression of $p16^{INK4A}$ in OSCC may be related to human papilloma virus (HPV)-induced OSCC. This concept has been drawn from the data obtained from intraepithelial and invasive squamous neoplasia of the uterine cervix, where the role of HPV has been widely demonstrated and the functional inactivation of pRb by the HPV oncoprotein E7 and consequent $p16^{INK4A}$ overexpression is found in almost all cases (Klaes, 2001; Klaes, 2002). Squamous epithelial $p16^{INK4A}$ overexpression may be present in the uterine cervix for many years before the development of squamous cell carcinoma, and $p16^{INK4A}$ detected by immunohistochemistry has become an important biomarker for cervical intraepithelial neoplasia (Klaes, 2001; Murphy, 2005) and useful in the differential diagnosis of squamous metaplasia (Regauer, 2007). Immunohistochemical techniques have recently been applied to oral lesions

with malignant potential, to determine whether $p16^{INK4A}$ is useful in identifying oral dysplasia, but the results were conflicting. A positive relationship between reduced $p16^{INK4A}$ protein expression and dysplasia in oral lesions has been reported (Papadimitrakopoulou, et al. 1997; Kresty, et al. 2002). By contrast, Gologan, et al. 2005 and Cunningham et al. 2006 demonstrated a positive correlation between $p16^{INK4A}$ expression and grade of dysplasia, whereas (Bradley, et al. 2006 and Buajeeb, et al. 2009) stated that $p16^{INK4A}$ is not helpful in differentiating dysplastic from non-dysplastic mucosa. $p16^{INK4A}$ overexpression has recently been proposed as a surrogate marker for HPV. Moreover, $p16^{INK4A}$ immunostaining and subsequent HPV-specific polymerase chain reaction (PCR) and⁄or fluorescence in situ hybridization (FISH) have been recommended for identifying OSCC with biologically associated HPV (Hafkamp, et al. 2008; Smeets, et al. 2007). To overcome this limitation, it has been proposed to couple p16 IHC with a secondary assay to directly detect HPV DNA or RNA (Thomas and Primeaux. 2011).

2.3.5.2- Cytokeratin-19:

Cytokeratin 19 (CK19), also known as keratin 19, is a type I intermediate filament protein with a molecular weight of approximately 40-44 kD. Cytokeratin 19 is a heterotetramer composed of two type I and two type II keratin subunits. Unlike other cytokeratins, cytokeratin 19 lacks a C-terminal non-helical extension. This cytokeratin is widely expressed in the periderm (transient superficial layer enveloping developing epidermis), muscle, intestine, bile duct, esophagus, stomach, and thymus. Cytokeratin 19 can be up regulated by vitamin A and is thought to play a critical role in embryogenesis. Cytokeratin 19 intereacts with the pinin protein and has been shown to be modified by phosphorylation (Ser10, Ser35).The A53-B/A2 monoclonal antibody recognizes human cytokeratin 19 and is useful for Western blotting. This

antibody has also been reported to be useful for immunoprecipitation, immunohistochemistry (paraffin sections), immuno-cytochemistry, and ELISA (Eckert, 1988).

Cytokeratin (CK) alterations have been reported in carcinomas and these have been associated with specific aspects of tumour behaviour. Immunohisto-chemistry with monospecific CK19 antibody was used to study the expression pattern in normal mucosa, dysplasias, and oral squamous cell carcinomas (OSCC). In non-keratinzed normal mucosa, CK19 was detected in the basal cell layer, while in dysplasias (diagnosed in H and E stained sections, mild-severe) stained strongly for CK 19 in the basal and supra basal cell layers indicating layer specificity for CK 19 expression. In OSCC, in the number of CK19 labelled cells increased from well to poorly differentiated tumour (Ram, et al. 2005). CK19 protein expression and gene transcription in OSCC tissue correlate significantly with pathologic differentiation grade. Positive CK19 expression in distant tissue suggests a higher tumor recurrence rate and a lower survival rate. Moreover, Increased CK19 expression in the suprabasal layer of oral mucosa is considered to correlate with premalignant change in oral epithelium (Nie et al 2002). However, there are still debates on the positive rate of CK19 expression in OSCC tissue. Vora, et al. (2003) found that the positive rate of CK19 protein was only 29% in tongue squamous cell carcinoma tissue. Hamakawa, et al. (1998) found the positive rate of CK19 protein was 66.7% in OSCC tissue, but only 24% was designated as strong staining intensity. The CK19 positive rate in OSCC tissue was 90.9% (30 out of 33) detected using IHC, which was significantly higher than that in distant tissue (15.2%, 5 out of 33) ($P < .001$) (Zhong, et al. 2007). Nie, et al. (2002) found a 100% positive rate of CK19 protein in OSCC tissue. Therefore, they decided to investigate the CK19 expression in both cancerous and

distant tissues from OSCC patients. CK19 is believed to be a putative marker for epithelial stem cells (Larouche, et al. 2005). Aberrant expression of CK19 has been reported in oral and cervical epithelial dysplasia and SCC (Zhong, et al. 2007; Takeda, et al. 2006). These findings indicate that oral epithelial dysplasia may be histologically recognized by the distribution disturbance of proliferating cells and stem cells within the stratified layers of the epithelium (Mittal, et al. 1992).

2.3.6- Molecular pathology:

2.3.6.1- Polymerase chain reaction (PCR):

The PCR is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Developed in 1983 by Kary Mullis, (Bartlett, et al. 2003) PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications (Saiki, et al. 1985; Saiki, et al. 1988). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used inforensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size (Cheng, et al. 1994). The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Carr & Moore, 2012).

2.3.6.2- Insituhybridization:

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (probe) to localize a specific DNA or

RNA sequence in a portion or section of tissue (in situ), or, if the tissue is small enough (e.g. plant seeds, Drosophilaembryos), in the entire tissue (whole mount ISH), in cells and in circulating tumor cells (CTCs) (Jin & Lloyd, 1997). For the samples that were found to be positive for HPV L1 by PCR, the HPV type was identified using catalyzed reporter deposition in situ hybridization (CARD-ISH) as previously described (Huang, et al. 1998). In situ hybridization assays for HPV DNA can provide data on the presence of HPV in different cells, but have limited sensitivity for certain HPV genotypes and cannot demonstrate oncogene transcription. Viral oncogene expression can be demonstrated by the polymerase chain reaction (PCR) technique, but this does not provide information about the viral load and the distribution of HPV DNA (Gillison, 2006). PCR combined with in situ hybridization can detect HPV-infected cells with low viral loads, and can also elucidate the distribution of HPV DNA within the tumour (Miller, et al. 1994).

Computed Tomography (CT): Is a medical imaging procedure that uses computer-processed X-rays to produce tomographic images or 'slices' of specific areas of the body. These cross-sectional images are used for diagnostic and therapeutic purposes in various medical disciplines. Digital geometry processing is used to generate a three-dimensional image of the inside of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation (Herman, 2009).

Magnetic Resonance Imaging (MRI): Is a medical imaging technique used in radiology to visualize internal structures of the body in detail. MRI makes use of the property of nuclear magnetic resonance (NMR) to image nuclei of atoms inside the body. MRI can create more detailed images of the human body than are possible with X-rays (Sheil, 2012).

Evaluation of the location and extent of squamous cell tumors can be done with both CT and MRI. CT has long been regarded as the imaging modality of choice for assessing the size, location, and spread (both in soft tissue and regional lymph nodes) of the primary tumor (Kalavrezos, et al. 1996). The MRI offers potential advantages in terms of superior soft tissue contrast, multiplanar imaging capability, lack of ionizing radiation, and freedom from metallic artifacts from dental restorations (Hermans, et al. 1994). In the mandible CT outperforms MRI in determining cortical involvement, but MRI is more reliable in evaluating the extension of the tumor into marrow (Huntley, et al. 1996). Ultimately, none of the imaging techniques are accuc determining the degree (rate enough by themselves, and a combination of clinical and multiple imaging techniques offer the best results in of osseous involvement (Ord, et al. 1997).

Management and Treatment: With completion of clinical staging of disease, that is the assimilation of all information from clinical examination, imaging studies, and pathology; the patient's data is ideally presented at an oral lesions conference or tumor board. Here it is reviewed by the multiple disciplines; oral lesions surgery, medical and radiation oncology, dentistry, pathology, radiology, speech therapy, nutrition, and social services. In this type of environment, the optimal and most comprehensive treatment plan can be formulated for this complex disease.

Prevention: The majority of OSCC is related to tobacco and alcohol use and hence the best way to prevent OSCC would be to educate the general population about the risk and to help them stop smoking. Furthermore, identifying individuals with inherited dysfunction in their DNA repair systems, cell cycle control and apoptotic pathways rendering them sensitive to tobacco induced carcinogenesis would have a great impact on prevention as well as early detection (Sturgis et al. 2004). In addition prevention of

HPV induced OSCC would include changes in sexual behaviour since each alteration of sexual partner increases the risk for HPV infection (Smith et al. 2004). Vaccine, against HPV induced tumors particularly cervical cancer have been developed and is currently being used (For HPV 16 and 18), it would also be of interest to evaluate their effect on HPV induced OSCC.

Prognosis and survival: The prognosis in oral cancer varies according to the specific intra-oral location. Local and regional lymph node involvements are the most important prognostic factors. An increasing risk is observed with larger sized tumors, while poorly differentiated tumors metastasize more frequently than well-differentiated tumors (Pindborg, 1980). Approximately half of people with oral cancer will live more than 5 years after they are diagnosed and treated. If the cancer is found early, before it has spread to other tissues, the cure rate is nearly 90%. However, more than half of oral cancers have already spread when the cancer is detected. Most have spread to the throat or neck (Posner, 2007; Wein, et al. 2010). Various molecular indicators of prognosis have been observed.

Chapter Three
Materials and Methods

3.1. Study design:

This is a prospective analytical case control study aimed to identify of HR-HPV subtypes, among patients with oral lesions in Khartoum State. The study was conducted in Dental health hospital of North Khartoum, Khartoum hospital, Omdurman hospital, Police hospital, Almubarak Histopathology Laboratory and National Center for radiotherapy and nuclear medicine, during the period from June 2010 to Decimber 2013.

3.2. Materials

Formalin- fixed, paraffin- wax (FFPW) processed oral lesions blocks as well as data concerning any specimen were retrieved from histopathology laboratories.

3.3. Study population:

Two hundred individuals were enrolled in this study; of whom 100 patients with oral cancer and 100 patients with non-neoplastic oral lesions, includinding all patients with oral lesions reffered to the distinct laboratories during the period (2007-2011).

3.4. Samples size:

Sample size was calculated according to the following equation: considering confidence interval 95±2, it was found to be 200 to study subjects.

$$ss = Z^2 * (p) * (1-p)$$

c^2

Z = Z value (e.g. 1.96 for 95% confidence level)

p = percentage picking a choice, expressed as decimal

(0.5 used for sample size needed)

c = confidence interval, expressed as decimal

3.5. Ethical consideration:

The study was submitted for approved by the Sudanese ethical committee from Dental health hospital of North Khartoum and Sudan University of Sciences and Technology**.**

3.6. Sample processing Methodology:

Tissue specimens were fixed in 10% neutral buffer formalin as life as possible and processing by the tissue processor machine for paraffin embedding. All samples were processed in paraffin wax and cut in Rotary microtome for H&E, Immunohisto-chemistry and DNA extraction for Polymerase Chain Reaction (PCR). From each block three sections were prepared.

3.6.1. Haematoxylin and Eosin:

First section (4**µ**) was stained using Haematoxylin and Eosin stain for histopathology diagnosis. The slides of oral lesion were evaluated for pathological changes under light microscope.

3.6.2. Immunohischemistry: ((ChemMateTM EnVision, + / HRP))

Second section (5**µ**) was stained with Immunohisto-chemical For immunohistochemical study, 5 µm thick sections were cut on poly-L-lysine-coated slides from paraffin wax blocks. Slides were dewaxed with xylene and rehydrated through graded alcohol then followed by blockage of endogenous peroxidase activity using 1% hydrogen peroxide solution in methanol for 30 min, and the slides were washed 3 times with phosphate-buffered saline PBS (pH 7.4), every three minutes, remove the sections, to get rid of dry tissue surrounding liquid (organization should not dry), flat on the wet box. For antigen retrieval, they were immersed in 1 mM citrate buffer (pH 6.0), and treated in a water path at 95°C for 30 minutes. The slides reacted with various pre-diluted primary antibodies overnight at 4°C in a humid chamber. The primary antibodies were used in this study are: monoclonal antibody cytokeratins 19 and $p16^{INK4A}$ respectively, *(ChemMate ™ EnVision, + / HRP /DAB, rabbit / mouse two-step staining).* The working fluid in the organization, and incubated for 30 minutes at room temperature, incubation is completed, wash the sections with PBS. Washed 3 times with PBS, every three minutes (organization should not dry), flat on the wet box. Dropping 50-100µl increased slightly A fluid *(ChemMateTMEnVision, of + / HRP),* the organization, and incubated for 30 minutes at room temperature. Incubation is completed, the slide placed in PBS buffer, washed 3

times, every 3 minutes (organization should not dry), flat on the wet box. Added to prepare a good chromogenic reagent DAB working solution 50-100 micro-open, snapped temperature and incubated for 5-10 min and to control the color under the light microscope. The color is complete, rinse with distilled water to terminate color. The slides were then rinsed in tap water, counterstained in Mayer's haematoxylin and mounted in synthetic mountant. A positive reaction was seen as brown granular cytoplasmic staining. Percentage positivity was calculated in each case as the number of positive cells for every 500 cells counted (Hsu, et al. 1981).

Evaluation of immunostaining: The expression of CK 19 and $p16^{INK4A}$ were determined independently by three oral pathologists. A section was scored according to the staining intensity and staining area. The staining intensity were scored as, no staining (score 0), light yellow (score 1), yellow to brown (score 2), and dark brown (score 3). The staining area were scored as, no staining (score 0), positive staining for less than one-third of tissue section (score 1), positive staining area ranged from one-third to two-third of tissue section (score 2) and positive staining for more than two-third of tissue section (score 3). Sections were considered negative or positive according to the sum of above two scoring systems and a score ≥3 was regarded as positive. Ten high-power fields were randomly selected for observation. Percentages were calculated to determine the expression of these markers.

All the p16 AND CK19 immunostained sections were reviewed and assessed for the staining patterns according to the following parameters:

a) Localization of staining cells in relation to layers of squamous epithelium: basal, intermediate, or superficial;

b) Distribution of staining cells: focal/scattered or diffuse and confluent/continuous;

c) Staining intensity: weak, moderate, or strong.

All tests were performed two-sided, and p-values below 0.05 were considered statistically significant.

3.6.3- DNA extraction:

Third section HPV-DNA extraction of paraffin blocks. Three (25 µm) sections were cut from each paraffin block and put into Eppendorf tubes. Sections were cut using a clean pair of gloves for each block. The microtome blade, chuck and knife holder were thoroughly cleaned with xylene and rinsed with alcohol between sectioning of the blocks. All forceps and utensils used were also cleaned in this manner. Sections from different blocks were each cut using a new unused area of the microtome blade to prevent contamination from the blade. Each microtome blade was therefore divided into halves. The sections were treated with xylene to remove the paraffin wax followed by absolute alcohol. The tissue pellets recovered were suspended in ATL buffer *(Beijing Aide Lai Biotechnology Co., Ltd.*) and digested with Proteinase K (Aidlab Biotecim©) at 56°C for 3 hrs.

DNA was extracted using DNAse. The necessary precautions were taken to prevent contamination throughout the procedure.

DNA extraction of the tissue sections of the cases studied and all the oral tissue control blocks were carried out in the same order as in which they were sectioned. The extracted DNA was frozen at −20°C until PCR detection of HPV-DNA commenced.

3.6.3. Polymerase chain reaction (Genotyping of HPV):

3.6.3.1- Amplification of HPV

Type specific primers 12 types of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), were used to detect high risk in oral benign and malignant lesion. Amplification was performed according to HPV kit from *Sacace technologies- Casera – Italy*. The final reaction volume of 40 µl containing 20µl mix-1(contained in PCR tubes), 10 µl of mix-2 and 10 µl of extracted DNA (sample). Negative control, positive of high risk HPV DNA tubes contained 10 µl of DNA buffer, 10 µl of high risk HPV DNA. Samples and controls were amplified using Gene Amp PCR system 9700. The PCR program was described in Table 1.

3.6.3.2- Gel-electrophoresis:

Agarose Gel-electrophoresis: The PCR products were visualized in 2% Agarose gel with 0.5 µg/ml Ethidium bromide staining. The gel was prepared by dissolving 0.7 gm of agarose powder in 35 ml of 1X TBE buffer and heated at 65°c until the agarose completely dissolved, then left to cool at room temperature and 2 µl ethidium bromides was added. The comb was then placed appropriately in the electrophoresis tray and then gel was slowly poured and left to

set for 30 min for solidification .In a clean Eppendorf tube 10 µl of 1000bp DNA ladder and PCR product was loaded on the gel. Gel-electrophoresis was performed at 120V and 36 Am for 60 minutes. Pictures were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

3.6.3.3- Interpretation of PCR results

According to manufacture high risk HPV kit (from Sacace technologies- Casera –Italy) manual, the PCR product length for HPV16 should be 457 bp, HPV 18 should be 322 bp, HPV 31 should be 263 bp and HPV 33 should be 398 bp.

4.7. Statistical analysis:

The data will be analyzed by using (SPSS 11.0). The significance of differences will be calculated by using student *Chi-square test*.

P<0.05 will considered statistically significant.

Chapter Four

Results

In this study, 200 (100 with Oral cancer and 100 were with non-neoplastic oral lesions) patients with oral lesions were investigated for the presence of HPV infection. Their ages ranged from 4 to 85 years with a mean age of 43 year. The frequencies of patients with oral cancer were increasing with the increase of age. Hence, those with benign oral lesions, the frequencies of ages of patients were decreasing with the increase of age, as shown in Fig.2.

Table3 describes the distribution of the study population by the site of lesion and gender. A high frequency of oral lesions was seen among patients with buccal mucosa lesions, Constituting 71(35.5%); of whom 41(58%) were males and 30(42%) were females, followed by the salivary glands 46(23%); of whom 24(52%) were males and 22(48%) were females, Patients with tongue lesions represented 27(13.5%); of whom 19(70%) were males and 8(30%) were females. Oropharynx lesions were found in 21(10.5%) patients, of whom 16(76%) were males and 5(24%) were females. Sub-mandibular 15(7.5%); of whom 6(40%) were males and 9(60%) were females. Jaw lesions were found in 15(7.5%) patients, of whom 5(33.3%) were males and 10(66.7%) were females. Gingival lesions were seen in 5(2.5%) patients, of whom 1(20%) was males and 4(80%) were females.

Distribution of the study population by site of lesions and gender is shown in Fig.3. Males to females ratio was 1.2:1.0. Both females and males have a relatively similar distribution amongst benign lesions (50 cases, 50 cases); however, malignant lesions were more frequently seen among males compared to females (62 to 38 cases) respectively.

The distribution of the study subjects by histopathology and gender are described in Table.4. The great majority of oral cancers are OSCCs variety followed by adenocarcinomas, mucoepidermal carcinomas and rhabdomyo-sarcomas constituting 90/100(90%), 4/100(4%), 4/100(4%) and 2/100(2%), respectively. Benign lesions were predominated by inflammation followed by, pleomorphic adenoma, squamous papilloma, pyogenic granuloma, reactive hyperplasia, dysplasia, ameloblastoma, hyperkeratosis, haemangioma, fibroma and lingual colloid goiter constituting 37/100(37%), 24/100(24%), 7/100(7%), 7/100(7%), 6/100(6%), 5/100(5%), 3/100(3%), 3/100(3%), 3/100(3%), 3/100(3%), 2/100(2%), respectively).

Males have higher frequencies of some malignant lesions as compared to females; squamous cell carcinoma (49% vs. 39.7%), adenocarcinoma (3.5% vs. 0%), and mucoepidermal carcinoma (2.6% vs. 1.1%), while rabdomyosarcoma were detected more commonly in females (2.3 Vs. 0%). Benign lesions have higher frequencies in males compared to females; pleomorphic adenoma (12.5% vs. 11.3%), squamous papilloma (4.5% vs. 2.3%), pyogenic

granuloma (4.5% vs. 2.3%), lingual colloid goiter (1.8% vs. 0%), hyperkeratosis (1.8% vs. 1.1%) and fibroma (1.8% vs. 1.1%). On the other hand, inflammatory changes whether acute or chronic were common in females compared to males (26.1 Vs. 12.5), reactive hyperplasia (4.3 Vs. 2.6), ameloblastoma (4.3 Vs. 0%), dysplasia (4.3 Vs. 1.8%), haemangioma (2.3 Vs. 1%).

Distribution of age, gender, site and types of oral lesion by HPV genotyping were indicated in Table 5. HPV genomic materials using A6 and A7 primers were detected in 12/200 (6%) of oral lesions. Of these, 6/12 (50%) HPV-16, 4/12 (34%) HPV-18, 1/12 (8%) HPV-31, and 1/12(8%) HPV-33. Out of the 12 HPV; 8/12(66.7%) HPV were found in malignant lesions, whereas, 4/12(33.3%) HPV were found in benign lesions. Consequently, the risk associated with HPV infection was found to be statistically significant ($P<0.001$).

The descriptions of the site of lesions are described in Table 5. The majority of malignant lesions were originating from buccal mucosa and oropharynx, particularly, SCCs, hence, most of benign lesions were seen in the salivary glands. Notably, most of the HPV positive lesions were found in tongue (25%) and jaw (25%), followed by buccal mucosa (16%), oropharynx (16%), salivary glands (8%) and gingiva (8%). Of the six HPV-16, two were detected in the buccal mucosa, two in the tongue, one in the oropharynx and the remaining one was detected in the jaw. The four positive cases of HPV-18, were detected in the salivary glands, tongue, jaw and gingiva, one for each, hence, the only one case of HPV-31 was

detected in the jaw and the remaining one case of HPV-33 was detected in the oropharynx. Seven (58%) HPV positive were found among males and the remaining 5 (42%) were found among females. Four HPV-16, two HPV-18 and one HPV-33 were detected among males, whereas, two HPV-16, two HPV-18 and one HPV-31 were detected among females.

The age group 40-49 years was the most susceptible to HPV infection, followed by 30-39, 50+ and <20 constituting 5/12(41.6%), 3/12(25%), 3/12(25%) and 1/12(8.4), respectively. The predominant isolated HPV genotypes were HPV16 (6 cases), flowed by HPV18 (4 cases), HPV31 (one case) and HPV33 (one case) genotypes, in this order.

The expression of p16ink4a and cytokeratin 19 was investigated in 200 of the studied cases. From the total 84(42%) patients were found to be positive for p16ink4a, while 74(37%) patients were detected positive for CK19, as shown in Fig.4.

Correlation between p16ink4a and genotyping of HR-HPV in oral lesions, the total number of cases detected by the PCR HPV genotyping were 12 cases, out of these 11(91.7%), were detected by p16, as shown in Table 6.

Regarding the p16ink4a with grade of oral squamous cell carcinoma, of whom (47%) were diagnosed as well differentiated and (40%) were moderately differentiated; while (54%) were poorly

differentiated squamous cell carcinoma. Furthermore, (75%) adenocarcinoma, (25%) mucoepidermal carcinoma and (100%) rhabdomyo-sarcoma $P < 0.0001$, as shown in Fig.5.

Distributions of the site of lesions by p16ink4a are described in Table 7. High frequencies of p16ink4a positive were found in buccal mucosa, followed by salivary glands, tongue, jaw, oropharynx, gingival, sub-mandibular and representing, 29/84(35%), 18/84(21%), 13/84(15%), 10/84(12%). 9/84(11%). 3/84(4%), 2/84(2%) in this order.

Distribution of the study population by Gender and p16ink4a are demonstrated in Fig.6. From the total cases recruited in this study, 84 patients were found to be positive for p16ink4a, out of these 49(58.3%) cases were males and 35(41.7%) were females.

Table (1): Show PCR Program used for amplification of HPV genes

Table (2): Sequences of type-specific PCR primers used in this study.

HPV-genotype	Sequence (5′-3′)	Amplification (bp)
16	CAC AGT TAT GCA CAG AGC TGC	457
18	CAC TTC ACT GCA AGA CAT AGA	322
31	GAA ATTGCATGA ACT AAGCTCG	263
33	ACT ATA CAC AAC ATT GAA CTA	398

Table (3): Distribution of the study population by site of lesion and gender.

Total	Gender		Site of lesion
	Female	Male	
46	22	24	Salivary gland
71	30	41	Buccal mucosa
15	9	6	Submandibular
27	8	19	Tongue
21	5	16	Oropharynx
15	10	5	Jaw
5	4	1	Gingival
200	88	112	Total

Table (4): Distribution of the study subjects by histopathology and gender.

Total	gender				Category	Variable
	female		male			
	%	No.	%	No.		
90	39.7	35	49	55	Squamous cell carcinoma	**Malignant**

4	0	0	3.5	4	Adenocarcinoma	
4	1.1	1	2.6	3	Mucoepidermal carcinoma	
2	2.3	2	0	0	Rhabdomyo-sarcoma	
24	11.3	10	12.5	14	Pleomorphic adenoma	**Benign**
37	26.1	23	12.5	14	Inflammation	
7	2.3	2	4.5	5	Squamous papilloma	
6	4.3	3	2.6	3	Reactive hyperplasia.	
7	2.3	2	4.5	5	Pyogenic granuloma.	
3	4.3	3	0	0	ameloblastoma.	
2	0	0	1.8	2	Lingual colloid goiter.	
5	4.3	3	1.8	2	Dysplasia.	
3		1	1.8	2	Hyperkeratosis.	
3	2.3	2	1	1	Haemangioma.	
3	1.1	1	1.8	2	Fibroma.	
200	100	88	100	112		Total

Table (5): Distribution of age, gender, site and types of oral lesion by HPV genotyping.

Total	**HPV genotyping**	**Category**				
	33.00	31.00	18.00	16.00		
1	0	0	1	0	< 20 years	**Age G**
0	0	0	0	0	21-29	
3	0	0	2	1	30-39	
5	1	0	1	3	40-49	
3	0	1	0	2	50+	

7	1	0	2	4	Male	**Gender**
5	0	1	2	2	Female	
1	0	0	1	0	Salivary gland	**Site of lesion**
2	0	0	0	2	Buccal mucosa	
3	0	0	1	2	Tongue	
2	1	0	0	1	Oropharynx	
3	0	1	1	1	Jaw	
1	0	0	1	0	Gingiva	
8	1	1	1	5	Squamous cell carcinoma	**Types of lesion**
1	0	0	1	0	Pleomorphic adenoma	
2	0	0	1	1	Inflammation	
1	0	0	1	0	Reactive hyperplasia	

Table (6): Correlation between p16^{ink4a} and genotyping of HPV.

Total	p16ink4a		Genotyping of HPV
	Negative	Positive	
188	115	73	.00
6	1	5	16.00
4	0	4	18.00
1	0	1	31.00
1	0	1	33.00
200	116	84	Total

Table (7): Distribution of the site of lesions by $p16^{ink4a}$.

Total	$p16^{ink4a}$		Site of lesion
	Positive	Negative	
46	18	28	Salivary gland
71	29	42	Buccal mucosa
15	2	13	Submandibular
27	13	14	Tongue
21	9	12	Oropharynx
15	10	5	Jaw
5	3	2	Gingival
200	84	116	Total

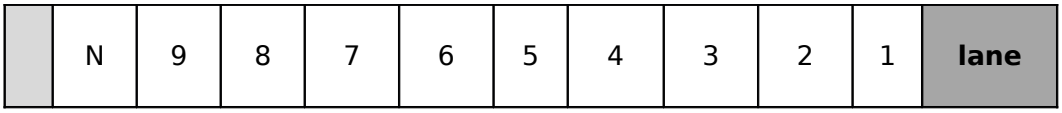

Fig.1. PCR amplification of high risk Oral lesions samples. The products were electophoresed on 2% agarose gel and stained with ethidium bromide. Lane (N) : Negative control, lane 1-,2,5,6,7.9. positive tumor samples, lane 1,2,5 HPV 18 positive tumor samples. Lane 3,4,8 negative samples. Lane 6 HPV 33. positive tumor samples. Lane 7 , 9 HPV 16 positive tumor samples

Fig.2. Description of the study population by age

Fig.3. Distribution of the study population by oral lesions and gender.

Fig.4: Correlation between by p16^{ink4a} and CytoKeratin 19.

Fig.5. Detection rate of cancer types by p16 ^{ink4a}

Fig.6. Distribution of the study population by Gender and p16^{ink4a}.

Fig.7: Upper Jaw, Well Differentiated Squamaus Cell Carcinoma 10x P16 immunohistochemical staining: Positive

Fig.8: Cheek, Well Differentiated Squamaus Cell Carcinoma 10x Ck19 immunohistochemical staining: positive.

Chapter Five
Discussion

Oral cancer is the tenth common cancer in men and fourteenth for both sexes in the world in terms of number of cases, accounting for approximately 480,000 new cases and mortality of 275,000 per year. In Sudan the incidence of oral cancer is 1547 and the mortality is 929 per year and the incidence rate was 13.5%, the mortality was 13% and the males' females' ratio was 1.3:1 (GLOBOCAN, 2008).

In the present study, the great majority of oral cancers are OSCCs (90%), which is similar to the other part of the world (Choi and Myers 2008). These findings were in agreement with the study reported, by Markopoulos (2012), the OSCCs is the most common malignant epithelial neoplasm affecting the oral cavity. A similar study by Choi and Myers (2008), reported that more than 90% of all oral neoplasms are OSCC. According to Globocan report 2008, the incidence of oral cancer in Sudan was high

compared to other parts of the world e.g. in Egypt is 6.4%, Ethiopia 11.6%, Chad 6.7%, Europe 5.5%. In the United States it is 4.5%, in India 19.9% and in Australia it is 4.2% (Globocan, 2008). However, the exact mechanism behind this variation is not well defined but it might be due to geographic variation and various etiological risk factors.

In Sudan, there are considerable numbers of oral cancers; most of them are attributed to the use of toombak. In very recent study by Ahmed, 2013, reported that, Toombak use and infection with high risk Human Papilloma Virus (HPV) were extensively investigated and linked to the aetiology of oral cancer.

Earlier studies demonstrated no association between Low risk HPV types (6 and 11) and oral cancers (Ibrahim, et al.1999). However, a recent study investigated the relationship between oral cancer and HR-HPV mainly type 16 and 18 showed strong association between these virusis and oral cancer (Ahmed and Eltoom, 2010). A similar study conducted by Ginawi, et al. (2012) reported that out of the 50 patients with OSCCs, 10 (20%) were found positive for HPV types 16 and 18 of which 50% were detected among males and 50% were demonstrated among females. The ten positive findings included 5 were HPV type 16, four were type 18 and one was positive for both HPV types 16 and18. The findings of our study were relatively lower than the results reported in the study by Ahmed and Mahgoob, (2007); Ginawi, et al. (2012), which might be due to their small sample size.

In the present study, HPV genotype was detected in 12/200(6%) of oral lesions (Malignant 8/12(66.7%), and Benign 4/12(33.3%)). The difference between the two lesions was found to be statistically significant ($P<0.001$). The presence of HPV in oral cancers was also investigated by Brew, et al. (2012). They assessed 17 cases of oral cancers and identified HPV in 8/17 (32.4%) of the cases. In Sudan a similar

study reported a 15% frequency rate ($P < 0.05$), Ahmed and Eltoom (2010). Furthermore, different rates were reported from different countries. Chau, et al. (2011) assessed 53 cases of oral cancers for HPV infection and found 17/53 (32%) were positive. Their findings might be attributed to their geographic variation and small sample size.

In this study the P16 and CK19 expression as indicators of preinvasive oral lesions was reported in 42.5% and 37.5% of the studied cases respectively, and the association between the two expression is statistically significant, Pvalue=0.002, and to the best of our knowledge this is the first report in this context from Sudan. Moreover, from this study, 44% (39 out of 90) of OSCC tissues were found positive for CK19 protein, while 46% (41 out of 90) of OSCC tissues were found positive for p16 expression protein, which agrees with the high expression of CK19 protein in OSCC tissue (Nie, et al. 2002).

Cytokeratin (CK) alterations have been reported in carcinomas and these have been associated with specific aspects of tumour behaviour. Immunohisto-chemistry with monospecific CK19 antibody was used to study the expression pattern in normal mucosa, dysplasias, and oral squamous cell carcinomas (OSCC). In non-keratinzed normal mucosa, CK19 was detected in the basal cell layer, while in dysplasias (diagnosed in H and E stained sections, mild-severe) stained strongly for CK 19 in the basal and supra basal cell layers indicating layer specificity for CK 19 expression. In OSCC, the number of CK19 labelled cells increased from well to poorly differentiated tumour (Ram, et al. 2005). CK19 protein expression and gene transcription in OSCC tissue correlate significantly with pathologic differentiation grade. Positive CK19 expression in distant tissue suggests a higher tumor recurrence rate and a lower survival rate. Moreover, Increased CK19 expression in the suprabasal layer of oral

mucosa is considered to correlate with premalignant change in oral epithelium (Nie et al 2002). However, there are still debates on the positive rate of CK19 expression in OSCC tissue. Vora, et al. (2003) found that the positive rate of CK19 protein was only 29% in tongue squamous cell carcinoma tissue. Hamakawa, et al. (1998) found the positive rate of CK19 protein of 66.7% in OSCC tissue, but only 24% was designated as strong staining intensity. The CK19 positive rate in OSCC tissue was 90.9% (30 out of 33) detected using IHC, which was significantly higher than that in distant tissue (15.2%, 5 out of 33) ($P < .001$) (Zhong, et al. 2007). Nie, et al. (2002) found a 100% positive rate of CK19 protein in OSCC tissue. Therefore, they decided to investigate the CK19 expression in both cancerous and distant tissues from OSCC patients. On another hand, in this study $P16^{INK4A}$ immunostaining was used to screen tissue samples obtained from patients with oral lesions to indicate the presence of HPV infection. The current study has indicated a significant association between HPV infection and oral lesions in Sudan ($P< 0.02$). Over expression of $p16^{INK4A}$ might specifically identify HPV infections that are biologically relevant in the carcinogenesis of oral squamous cell carcinomas and cervical carcinomas. However, it should be noted that expression of $p16^{INK4A}$ is not limited to HPV-positive tumors, and the use of this marker alone as an indicator of biologically relevant HPV infections inevitably entails the risk of including some HPV negative $p16^{INK4A}$ positive results (Lassen, et al. 2009). Furthermore, the association between $p16^{INK4A}$ expression and HPV infection has been firmly established in oropharyngeal SCC (Arafa, et al. 2008; Robinson, et al. 2010).

The assessment of p16 is carried out in oral lesions, a considerable numbers of cases (37.5%) that are positive for CK19 can also be (42.5%) positive for P16, therefore, the highest number of cases that are p16 positive are indeed seen in those disclosed 90%

positivity for CK19. However, the goal of this relation should be around the clinical outcomes, since, CK19 is indicator for worse and P16 is a predictor for better prognosis.

The study also shows that loss of CK19 expression and p16 overexpression are significantly correlated with oral cancer staging, suggesting that both of the activities may play an important role in oral cancer development and progression.

But the exact role of CK19 in the genesis of HPV remains unknown.

The result of $p16^{INK4A}$ mentioned above, the present study in accordance with reports mentioned by Smith, et al. (2010), $p16^{INK4A}$ may serve as a surrogate biomarker of oncogenic HPV infection and most of the documented evidence showing a better prognosis in $p16^{INK4A}$ positive SCC was associated with HPV infection. Another study by Lucio, et al. (2010), showed that the high positive and negative predictive values of p16 expression make it highly likely that a p16-positive OSCC is preceded by p16-positive lesions. In a very recent study by Gaurav, et al. (2013), in which they assessed the HPV 16 positive cases, more than 60% of cells were positive for $p16^{INK4A}$ IHC in oral epithelial dysplasias (OED) (50%) and OSCC (85.71%). A number of explanations might be considered for this decoupling of $p16^{INK4A}$ accumulation and HPV positivity. First, there is the possibility that infection with other viruses contributes to the observed $p16^{INK4A}$ overexpression by functionally inactivating pRb in a similar way as the HPV oncoprotein E6, E7, and pushing infected cells into proliferation. Second, $p16^{INK4A}$ accumulation might be the result of cellular senescence and／or ageing. It has recently been suggested that a combination of p16, together with in situ hybridisation and PCR for HPV would be required for accurate characterisation (Thavaraj, et al. 2011).

In this study, the genotyping of the infected specimens using PCR revealed the presence of high risk HPV subtypes 16, 18, 31 and 33. HR-HPV 16 was detected in 6 (50%) cases, HPV 18 in 4 (34%) cases, and the remaining types HPV31, HPV 33 were found in only one (8%) case for each. These findings are consistent with a study by Wei, et al. (2012), which reported that, HPV16 is the most common genotype in oral lesions, in which HPV16 and 18 are the most prevalent HPV genotypes. Another recent study by Brew, et al. (2012), reported that, HPV 16 in 6 cases (75.0%), co\infections HPV 16/18 and 16/33 in one case each (12.5%). According to disagreement between the results of these studies and the others, that reported by Jenice, et al. (1998), they found HPV-16 was detected in 15 out of 100 (15%) oral tumours, 27 out of 80 (34%) potentially malignant lesions and 15 out of 48 (31%) of the corresponding normal mucosa in the patients with oral lesions. HPV-18 was not detected in any of the oral cancers, oral lesions and normal mucosa. HPV-33 also not detected in the oral cancers, oral lesions and corresponding normal mucosa. However, and to the best of our knowledge, no previous studies that have investigated the four HPV genotype 16, 18, 31 and 33 in Sudan. These findings were in agreement with the study reported by Nasman, et al. (2009) in biopsies from Indian patients with the premalignant lesion where they found a high prevalence of HPV (91%) and of these 55% were of the high risk HPV-16 type. In the oropharynx, HPV16 accounted for the overwhelming majority of HPV-positive cases (86.7%). The other oncogenic HPV types 18 and 33 are also detected in invasive cervical cancer biopsies were also detected in HNC biopsies (Kreimer, 2005; Termine, 2008). Montaldo, et al. (2007) reported only two HPV genotypes (HPV 16 and HPV 31) were detected in OSCCs. HPV16 was the most common type present and was detected in 16.0% of OSCC, accounting for almost 70% of HPV positive cases. HPV18 was the next most common

oncogenic HPV type, detected in 8% of OSCC (Kreimer, et al. 2005; Adelstein, et al. 2009). Moreover, HPV16 was the predominant genotype present, which is consistent with other studies (Goon, et al. 2009; Ang, et al. 2010; Machado, et al. 2010).

In this study, compared PCR and $p16^{INK4a}$ IHC for the assessment of HPV status in oral lesions. HPV was detected using PCR method in 11cases out of 12 cases that expressed P16 positive, whereas only one case was negative for P16. The detection of HPV using PCR was statistically significantly compared with P16 expression P-value ≤0.005. Nevertheless, $p16^{INK4a}$ staining was reported to be both an efficient for HPV surrogate marker and has good clinical outcome correlates for tonsillar SCCs (Kua, et al. 2008; Wittekindt, et al. 2005). However, as with the present study, HPV negative/$p16^{INK4a}$ positive or HPV positive/p16INK4a negative specimens were reported (Wittekindt, et al. 2005; Kua, et al. 2008). A study that included 26 $p16^{INK4a}$ positive but HPV negative by PCR in oropharyngeal tumors found that these patients had a better survival than patients that were negative for $p16^{INK4a}$ and HPV, suggesting the possibility that $p16^{INK4a}$ immunohistochemistry alone may be sufficient for risk stratification in oropharyngeal SCC (Lewis, et al. 2010). In our study, we also found cases that were HPV positive by PCR and yet $p16^{INK4a}$ negative. This could occur as a consequence of mutation, deletion, abnormal microDNA expression, or epigenetic events impacting the p16 gene; for example, smoking may increase the risk of p16 methylation (Ma, et al. 2011).

The clinical significance of non-alcohol/tobacco related HPV negative/$p16^{INK4a}$ positive OSCC, PCR HPV positive samples that are $p16^{INK4a}$ negative, and, PCR HPV positive samples that are p16 INK4a negative remains to be fully resolved.

In the present study, most of the positive samples were identified in tongue, jaw and oropharynx sites. These findings support another studies conducted in many countries

which detected the presence of HPV in many sites of oral region particularly tongue and oropharynx. The most systematic review found that the average HPV DNA positivity was 35.6% for oropharyngeal cancer and 23.5% for oral cavity cancer (Kreimer, et al. 2005). In a study reported by Guily, et al. (2011) found that, the HPV prevalence was 46.5% in oropharyngeal carcinomas and 10.5% in oral cavity carcinomas. However, the most frequent anatomical sites were tonsil (58.9%) and base of tongue (13.7%), for the oro-pharynx and floor of mouth (41.1%) and oral tongue (38.3%) for the oral cavity (Guily, et al. 2011). In a recent study by Brew, et al. (2012), HPV 16 was the most prevalent type and was found in 89.7% and 95.5% of HPV-positive oro-pharyngeal and oral cavity carcinoma cases, respectively. On the other hand, the most common site of oral cancer used to be the body of the tongue, representing 40% of all diagnosed OSCC in the study of Neville and Day (2002); Marur, et al. (2010) reported the observation of an increasing incidence of SCC, specifically in the tonsil and tongue base in USA. In other study, 35.3% of OSCC were located in the tongue, supporting these findings. Moreover, HPV positivity was significantly higher in oropharyngeal tumors, especially of the tonsils, than in oral cavity tumors and was increased, although not significantly, in tumors with lower histological differentiation and in male patients. Generally, other studies have recognized significant associations between HPV and white race and younger age (Smith, et al. 2010). In a recent study reported by Brew, et al. (2012), samples were collected exclusively from oral cavity cancers. Besides the tongue being the most prevalent site of tumors, in HPV positive cases the tumor was located most frequently in the mouth floor (3/8) with only one located in the tongue. In a study conducted by Lee, et al. (2010) on the relevance of HPV in oral tongue SCC, the authors observed that HPV-16 was the most common type associated with a prevalence rate of 85%.

The incidence of oral cancer has been reported to increase in age. In this study, the peak incidence of oral cancer was observed in the age of 40-49 years. The reasons for this remain unclear, but it might be due to the cumulative effects of long time exposures to carcinogens, the failings of DNA repair mechanisms and aging (Horstmann, et al. 2008). These findings were similar to studies conducted by Ahmed and Mahgoob (2007); Ginawi, et al. (2012), they found that 86.6% of oral cancers among Sudanese patients occurring in males with mean age of 48 years. In another recent study, oral HR-HPV infections showed a bimodal age pattern, with peak prevalence at 30–34 year of age and another peak at 60–64 year of age (Gillison, et al. 2012). According to literature, among different oropharyngeal cancers, OSCC is the most incident (90–95%), predominantly in male older than 60 years (Neville and Day, 2002; Pintos, et al. 2008). However, in one study they found that patients under the age of 20 years represented approximately 0.8% of oral cancer, 3% in 20 to 34 years, 8% in 35 to 44 years, 20% in 45 to 54 years, 23% in 55 to 64 years, 23% in 65 to 74 years, 17% between 75 to 85 and 5.8% in 85 years of age and older (Nelson, 2005). A study in Indian population reported maximum cases (54%) of oral cancer occurred in age group of 55-74 years (Napier and Speight, 2008).

In the present study, much higher incidence of tumors was noted among males compared to females. The variation of incidence of oral cancer in gender may be due to the variation in the environmental, dietary exposures, innate sexual characteristics and tobacco chewing, smoking and alcohol intake are higher in men than women; it is also known that men acquire these habits earlier than women (Rahmani, et al. 2012). These findings are supported the study by Ahmed and Mahgoob (2007); Ginawi, et al. (2012) that men accounted for over 74%. Oral cancer in Sudan is lower among females. This is because toombak use (synergistic factor to HPV) is uncommon

among females, as it is considered as a social stigma in the Sudan. Furthermore, a previous study reported by Ibieta, et al. (2005) found more positivity in men than in women (71% vs 29%) in oral cancers in a Mexican population. They found no differences in HPV status among men and women with premalignant lesions. However, HPV-associated oropharyngeal cancers generally are diagnosed at slightly younger ages in men than in women. In another recent study, the prevalence of HPV16 was significantly higher in men than in women (1.6% vs 0.3%; $p < 0.00.1$), but no detailed genotype-specific data were presented among males (Gillison, et al. 2012). Furthermore, the detection of oral HPV DNA carriage in men is common (Kero, et al. 2012).This is partly ascribed infection with HPV, particularly type 16 and 18 which is increasing in those diagnosed with malignancy and they do not present the traditional risk factors. This new and rapidly growing younger group where males slightly outnumber females is infected with the same type of virus responsible for most cervical cancers (Globocan, 2008). It is also known that men acquire habits earlier than women.

The limitations of this study, patients were not interviewed therefore, the study lacks the correlation of symptoms and the pathological findings, and again it lacks the control group for risk association, for further evaluation. Also did not have knowledge about the patient's socioeconomic status, nutritional status, previous health history nor family relations, lack of information regarding alcohol intake and smoking habits. Such information would indeed be of interest to compare HPV prevalence in presence or absence of other risk factors.

In summary, these data reinforce the clinical and biological importance of HPV-associated OSCC in the Sudanese population and confirm that the rates of HPV-associated disease are similar to those reported elsewhere in the world.

Conclusion and Recommendations

1. Conclusion

The findings of the present study suggest the following conclusion:

1. the most prevalent oral cancers in Sudanese patients was squamous cell carcinoma which represents more than 90% of all HNCs

2. The majority of oral cancers in Sudan were found in population over 50 years old.

3. the most affected sites with oral cancers in Sudanese patients were oral cavity and oropharynx.

4. The presence of HPV in squamous cell carcinoma of the oral lesions in a significant number of cases. This strengthens the fact that HPV plays a role in the etiology of the oral cancer. Overall, the study found that there is association between HPV16, 18, 31 and 33 infections and OSCC in Sudan.

5. P16 immunohistochemical method is a useful and reliable method of identification of HPV in tissues from OSCCs.

2. Recommendations

Further studies especially multicenter studies with large study population are recommended to measure the real burden of HPV in etiology of oral cancers in Sudan, this in addition to provide information about different genotypes. P16 immunohistochemical method can be used alone as a detection technigue for HPV in oral cancers but it is better to be combined with molecular methods. Further studies should be done including the factors of patients such as socioeconomic status, nutritional status, previous health history family relations, lack of information regarding alcohol intake and smoking habits.

Chapter Six

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