Chapter One
Introduction

Oral cancer (OC) is the sixth most common cancer in the world. Each year approximately 270,000 cases of oral cancer are diagnosed worldwide and 97,919 patients die annually (Mortazavi, *et al.* 2014). In 2014 an estimated 42,440 American new cases of cancer of the oral cavity with approximately 8,390 deaths (American Cancer Society, 2014). Oral cancer has the highest rates of incidence in Western Europe, India, South Africa and Australia. There is a particularly high incidence of oral cavity cancer in males in France whereas in females (Ferlay*, et al*. 2010). In the countries of the European Union (EU), each year an estimated 43,847 new oral cancer cases were diagnosed. India has the world's highest incidence of oral cancer, with 77,003 new cases a year (IARC, 2012). Increases incidence of oral cavity and pharynx cancer have been reported in Germany, Denmark, Scotland, Central and Eastern Europe (Stewardt and Kleiheus, 2010), which is thought to be due to an increase in alcohol consumption (Swerdlow, *et al*. 2010). OC is also more common in developing countries with estimated cases of 130,933 in men (3.1% of all cancers in men) and 68,617 in women (1.8% of all cancers in women). It represents the seventh most common malignancy for men in developing countries with an estimated mortality rate of 2.5% (IARC, 2012). 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 (Globalcan, 2002; Osman, *et al*. 2012). The incidences rate of OC in Sudan are 3.8% for men and 2.3% for women with prevalence is 3.2% (Ahmed, 2013). Many risk factors were well established in etiology of oral cancer, most of which related to lifestyle and environmental factors. Smoking and other tobacco useage are associated with about 75 % of oral cancer

cases; tobacco causes irritation of the mucous membranes of the mouth. Alcohol use is another high-risk activity associated with oral cancer. It is known to have a strong synergistic effect on oral cancer risk when a person is both a heavy smoker and drinker. Their risk is greatly increased compared to a heavy smoker, or a heavy drinker alone) Warnakulasuriya, *et al*. 2008). A study in Australia, Brazil and Germany point to alcohol-containing mouthwashes as also being etiologic agents in the oral cancer risk family. Constant exposure to these alcohol containing rinses, even in the absence of smoking and drinking, lead to significant increases in the development of oral cancer (La Vecchia*, et al*. 1997). Another potent risk factor is Human Papilloma Viruses (HPVs), particularly; high risk (HR) types 16 and 18. Many studies have strongly proved the link between HPV and oral cancer (Boyle, *et al*. 2008; Kreimer, *et al.* 2005). 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 5.2×10^6 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, 2009). HPVs are a group of more than 120 related viruses. Most HPV types cause warts on various parts of the body, but a few HPV types seem to be involved in some cancers. For example, nearly all cancers of the cervix are related to infection with certain HPV types. The current estimate is that HPV may be a factor in about one-fourth of oral and oropharyngeal cancers. According to the oncogenic potential, they are classified as low-risk and high-risk (Syrjänen, 2005). Both high-risk and low-risk

types of HPV can cause the growth of abnormal cells, high-risk HPV include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73 (Munoz, *et al*. 2003). It is important to note, however, that in the genital tract the large majority of high risk HPV infections regress on their own and do not cause cancer (Schiffman, *et al.* 2007). HPV must adhere to a specific receptor protein on the keratinocytes membrane. Once the virus entered into the cell, it transforms itself of its protein coat and the viral DNA may then utilize host cell themselves. These viruses elaborate early gene proteins (E) that are able to regulate the host cell cycle, or mitotic capabilities. The E6 and E7 proteins are most important in this respect; they bind two host proteins that are regulators of the keratinocytes at the time of cell division. E6 binds to a protein designated p53, a molecule that arrests cell division. However, once bound, it is degraded and this inhibition of keratinocytes mitosis is abrogated. Likewise, E7 binds a protein termed Rb; and, similarly, cell cycle regulation is troubled (Boyer, *et al.* 2009).

Early diagnosis in Oral cancer is vital as patient who present with early-stage disease has significantly better outcomes than those who present with late-stage disease. Routine physical examination, including a thorough oral examination, is the best way to detect oral cancer before they become symptomatic. Definitive diagnosis usually requires a biopsy. Additional information is obtained from a combination of imaging tests, such as Computed Tomography (CT), Magnetic Resonance Imaging (MRI) or Positron Emission Tomography (PET), endoscopy and fine-needle aspiration of any Oral mass (Ang, *et al.* 2010).

Definitive method for determining this is through biopsy and microscopic evaluation of the cells in the removed sample. A tissue biopsy, whether of the tongue or other oral tissues, and microscopic examination of the lesion confirm the

diagnosis of oral cancer, exfoliative cytology was thought of as a technique that could facilitate and accelerate clinical and histopathologic recognition of oral cancer. There are many techniques help in diagnosis of oral cancer include Fluorescent In Situ Hybridization (FISH), Southern Blot analysis, DNA microarray and immunofluorescent stain (Smith, *et al.* 2004). Many methods can be applied for diagnosis of oral cancer, as well as, identifying some related etiological agents such as HPV. HPV can be identified in cytological smears or biopsy using different techniques e.g. immunocytochemistry, polymerase chain reaction (PCR) and in situ-hybridization (ISH). On the basis of cytology and histopathology, HPV infection is characterized by koilocytosis, perinuclear cytoplasmic haloes, nuclear dysplasia, atypical immature metaplasia and binucleation. These methods show limited sensitivity and are unable to determine which types of HPV are involved in the infection of the epithelial cells (Smith, *et al.* 2004). ISH techniques employ the use of type-specific radioactively labeled DNA probes, which are complementary to HPV DNA sequences used for detection of viruses in the premalignant and malignant lesions of the oral cancer. ISH and immunohistochemistry have low sensitivity because these tests only detect the virus when it is present in more than 10 copies of the viral DNA per cell (Lee, *et al.* 2008). PCR is highly sensitive detection method for specific subtypes of HPV because it detects the virus in less than 1 copy of the viral DNA per cell (Tavares, *et al.* 2000).

Currently, the main treatment options for oral cancer are surgery, radiotherapy and chemotherapy. The types of treatment used will depend on the site and disease stage as well as on the patient's overall health status. For most early-stage tumors, surgery is carried out to remove the tumor. However, for certain anatomical sites, such as the base of the tongue, radiotherapy is used, either alone or combined with

surgery (Bernier and Cooper, 2005). Radiotherapy in combination with chemotherapy (most often cisplatin) - administered either as a definitive treatment or after surgery plays a role in the management of locally advanced and/or inoperable oral cancer; this is known as ‘radiochemotherapy’. Radiochemotherapy, however, is associated with significant toxicities. In addition, locally advanced oral cancer is associated with a poor prognosis due to high recurrence rates. For patients with advanced (metastatic) or recurrent disease, treatment options include systemic chemotherapy. Despite the introduction of chemotherapy treatment in this setting approximately 30 years ago, patients with advanced (metastatic) or recurrent oral cancer still have a poor prognosis, with median survival of 6-10 months (Bernier and Cooper, 2005).

1.2. Rationale

Oral cancer is the fifth most common cancer types in Sudan, the majority case of oral cancer are attributed to tobacco usage, alcoholic consumption and other environmental carcinogens. A study from Sudan has proved the significant role of HPV in the etiology of oral cancer (Ahmed and Eltom, 2010). Identification of these high-risk genotypes is very valuable in the management of oral carcinoma, both as a prognostic indicator and as a secondary screening test. However, early identification and intervention will probably have a significant impact on the reduction of oral cancer morbidity and mortality. Moreover, identification of HPV genotypes and its associate with mutation of tumor suppressor genes (p53, Rb) may help in the development of vaccination for HPV types. Many studies had been conducted to determine the most frequent HPV genotypes in different parts of the world, but there are no studies puplished from Sudan to determine the association of HR HPV and mutation of P53 and Rb genes in Sudan applying PCR and immunohistochemical techniques.

1.3. Objectives

- **General objective**
- To study the association of high risk HPV with immuno expresion of mutated p53 and Rb proteins among Sudanese patients with oral lesions.
- **Specific objectives**

1. To detect HR-HPV in patients with oral lesions by PCR
2. To determine the frequency of high risk HPV genotyping in oral lesions.
3. To detect mutations in p53 and Rb in oral lesions by using immunohistochemical method.

4. To identify the association of HR-HPV and (p53, Rb) genes mutations in oral lesions.

Chapter Two

Review of literature

Scientific background

2.1. Oral cavity:

The oral cavity extends from the lips to the palatoglossal folds and enclosed by the cheeks and lips and forms a slit-like space separating it from the gingiva and tooth (Snow, *et al*. 2009). The space bordered by the teeth and gingiva is the oral cavity proper. It is bounded inferiorly by the floor of the mouth and tongue and superiorly by the hard palate (Probst, *et al*. 2006). The buccal mucosa extends from the commissure of the lips anteriorly to the palatoglossal fold posteriorly (Szpirglas, 1999). It is lined by thick, non-keratinized stratified squamous epithelium and contains variable numbers of sebaceous glands and minor salivary glands (Miller, 2002). The duct of the parotid gland opens on a papilla or fold opposite the upper second permanent molar tooth (Szpirglas, 1999). The mucous membrane related to the teeth is the gingiva. The gingival mucosa surrounds the necks of the teeth and the alveolar mucosa overlies the alveolar bone and extends to the vestibular reflections. The junction between these two parts is marked by a faint scalloped line called the mucogingival junction which is non-keratinized or parakeratinized (Grays, 2005). The alveolar mucosa is reddish and covered by thin, non-keratinized stratified squamous epithelium (Ross and Pawlina, 2006). Minor salivary glands may be seen in the alveolar mucosa and occasionally atattached gingiva. The hard palate is continuous anteriorly with the maxillary alveolar arches and posteriorly with the soft palate. A median raphe extends anteriorly from this junction to the incisive fossa into which the nasopalatine foramen opens. Most of the palatal mucosa is firmly bound to the underlying bone forming a mucoperiosteum (Moore, *et al*. 2010;

Snow, *et al.* 2009). It is covered by orthokeratinized stratified squamous epithelium and posteriorly contains many minor mucous salivary glands (Ross and Pawlina, 2006). The oral part of the tongue (anterior two thirds) lies in front of the V-shaped sulcus terminalis. It is mobile and attached to the floor of the mouth anteriorly by a median lingual fraenum (Moore, *et al*. 2010). The dorsal part is covered by stratified squamous epithelium and contains several types of papillae. The most numerous are the hair-like filiform papillae which are heavily keratinized. There are less numerous and evenly scattered fungiform papillae which form pink nodules and contain taste buds. Taste buds here and in other oral sites are occasionally mistaken for junctional melanocytic proliferation or Pagetoid infiltration (Miller, 2002). In front of the sulcus terminalis there are 10-12 circumvallate papillae. These contain many taste buds on the surface and in a deep groove that surrounds each papilla. In addition, the ducts of minor serous salivary glands open in to the base of the groove (Moore, *et al*. 2010). At the postero-lateral aspect of the tongue where it meets the palatoglossal fold there are the leaf shaped foliate papillae. These also may contain taste buds on the surface and the core of the papillae often contains lymphoid aggregates similar to those in the rest of the Waldeyer ring. In addition, there are minor salivary glands in the underlying lingual musculature. The ventrum of the tongue is covered by thin, nonkeratinized stratified squamous epithelium which is continuous with similar mucosa in the floor of the mouth (Van and Staecker, 2006). Minor salivary glands (glands of Blandin and Nuhn) are present, predominantly towards the midline and deep within the lingual musculature. it can extend to involve the tip of the tongue (Moore, *et al*. 2010). The floor of the mouth is a horseshoe-shaped area between the ventrum of the tongue medially and the gingivae of the lower teeth anteriorly and laterally. It extends to the palatoglossal folds distally and

is in continuity with the retromolar pad behind the lower third molar tooth. The mucosa covers the major sublingual glands and the submandibular (Wharton's) ducts which open anteriorly onto the submandibular papillae on either side of the median sublingual fraenum (Moore and Catlin, 2000).

2.2. Inflammatory and infectious in oral cavity

2.2.1. Bacterial Infections

Streptococcal organisms are by far the most commonly isolated microorganisms from the oral mucosa. Caries of the dentition is a streptococcal infection. *Streptococcus viridans* is the most prevalent organism. Streptococcal infections appear as very erythematous, inflamed and painful lesions of the oral mucosa (Dahlen, 2009).

Gonorrhea; is caused by *Neisseria gonorrhoeae*. This disease takes on varying appearances within the oral mucosa. It can resemble acute necrotizing gingivitis exhibiting a necrotic pseudomembrane covering ulcerations or a severe erythematous inflammatory response of the oropharyngeal mucosa. The lesions are painful and cause difficulty in swallowing. When appearing as ulcerations, it can easily be mistaken for streptococcal infections or multiple aphthous stomatitis (Dahlen, 2009)**.**

Syphilis: It is a sexually transmitted infectious disease caused by the spirochete *Treponema pallidum*. It is almost always transmitted by sexual contact with infectious lesions, but it also can be transmitted in utero and through blood transfusion. 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).

2.2.2. Fungal Infections

Candidiasis is caused by the fungal species *Candida albicans*, which is common on the surfaces of the oral cavity, it is mostly seen in people with compromised immune systems and in diabetic patients. The incidence in patients with acquired immune deficiency syndrome (AIDS) is 40% to 90%. The lesions are white, slightly elevated, soft patches that consist mainly of fungal hyphae (Krishnan, 2012).

2.2.3. Viral Infections

Herpes simplex (HSV) Type I**;** is the most common viral that infect the lips and oral mucosa leading to mucosal lesions. The lesions are self-limiting and do not result in scarring of the tissues and frequently found on keratinized and non-keratinized epithelium, it recur when predisposing factors arise (Arduino and Porter, 2006).

Varicella Zoster Virus (VZV); is a herpesvirus that causes chickenpox. The virus often manifests in the oral, especially following primary infection. Intranuclear eosinophilic inclusions and ground-glass nuclear changes are seen in epithelial cells of the infected area (Talukder, *et al*. 2005).

Human Papilloma virus(HPV): It has been implicated in a variety of papillomatous and malignant squamous proliferations. 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 (Almadori, *et al*. 2002; Baez, *et al*. 2004).

2.3. Benign lesions of the oral cavity

2.3.1. Pyogenic Granuloma

This is a rapidly growing lesion that develops as a response to local irritation (e.g., poor hygiene, overhanging dental fillings), trauma, or increased hormone levels in pregnancy (Demir, *et al.* 2004). It is an erythematous, non-painful, smooth or lobulated mass that often bleeds easily when touched. Oral pyogenic granulomas most develop on the gingiva, but less common locations include the lip, tongue, and buccal mucosa (Sills, *et al*. 1996; Kroumpouzos and Cohen, 2001)

2.3.2. Palatal and Mandibular Tori

Tori are benign, non neoplastic, bony protuberances that arise from the cortical plate. They sometimes are mistaken for malignancies. These exostoses are considered developmental anomalies, although they usually do not appear until adulthood. A torus located along the midline of the hard palate is called a palatal torus, or torus palatinus, and a torus located along the lingual aspect of the mandible is called a mandibular torus, or torus mandibularis. Palatal tori are reported in 20 to 35 percent of the U.S. population, whereas mandibular tori are reported in 7 to 10 percent (Gonsalves,*et al*. 2007; Neville, *et al*. 2002). Removal is required only if a torus interferes with function or denture fabrication, or is subject to recurrent traumatic surface ulceration (Bouqout and Nikai, 2001).

2.3.3. Haemangioma

Haemangioma are the most common benign vasoformative tumours of infancy and childhood (Maaita, 2000). They are manifested within the first month of life, exhibit a rapid proliferative phase, and slowly involute to near complete resolution. There are

many ways to classify haemangiomas. According to Enzinger and Weiss, haemangiomas are broadly classified into capillary, cavernous, and miscellaneous forms like verrucous, venous, arteriovenous haemangiomas, and so forth. Capillary haemangiomas further include juvenile, pyogenic granuloma, and epitheliod haemangioma (Enzinger and Weiss, 2001).

2.3.4. Lymphangioma

Lymphangioma is a benign, harmatomatous tumour of lymphatic vessels with a marked predilection for the head and neck region. Oral lesions are most frequently found on the tongue and usually demonstrate a pebbly appearance as by their superficial location. Occurrence in other areas such as cheeks, lips, floor of the mouth, palate and gingiva has been reported (Neville, *et al*. 2002).

2.3.5. Mucocele

This is an area of mucin spillage in soft tissue resulting from rupture of a salivary gland duct. Children and young adults are most commonly affected, although this lesion may occur at any age. There is often a history of local trauma (e.g., biting). Many patients describe episodes of recurrent swelling with periodic rupture. The typical clinical presentation is a bluish, dome-shaped, fluctuant mucosal swelling (Gnepp, *et al*. 2001).

2.3.6. 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% 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).

2.3.7. Leukoplakia and Erythroplakia

Precancer and early oral cancer can be subtle and asymptomatic. A lesion may begin as a white, or red patch, progress to an ulceration, and later become an endophytic or exophytic mass. Oral leukoplakia, the best-known pre-malignant oral lesion, is defined by WHO as “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease. Analogous red lesions are called erythroplakia, and combined red and white lesions are known as speckled leukoplakia or erythroleukoplakia. Erythroplakia and speckled leukoplakia are more likely than leukoplakia to exhibit dysplasia or carcinoma microscopically (Boy, 2012).

2.4. Oral cancer

Oral cancer encompasses all the malignancies originating in the oral tissues, including cancers of the lip, tongue, gingiva, floor of the mouth, buccal mucosa, palate and the retromolar trigone.The most common type of the oral cavity is squamous-cell carcinoma (SCC), constituting about 90% of oral malignancies.It is described as an invasive epithelial neoplasm with varying degrees of squamous differentiation (Razavi and Sajadi, 2007).

2.4.1. Epidemiology of oral cancer

Oral cancer is the sixth most form of common cancer in the world each year approximately, 270,000 cases of oral cancer are diagnosed worldwide and 97,919 patients die annually (Mortazavi, *et al.* 2014). Oral cancer has the highest rates of incidence in America, Western Europe, India, South Africa and Australia (Ferlay*, et al*. 2010). In 2014, an estimated 42,440 American new cases of cancer of the oral cavity with approximately 8,390 deaths (American Cancer Society, 2014). In the countries of the European Union (EU), each year an estimated 43,847 new oral cancer cases were diagnosed. India has the world's highest incidence of oral

cancer, with 77,003 new cases a year (IARC, 2012). The region of the world with the highest incidence is Melanesia (31.5/100,000 in men and 20.2/100,000 in women). Rates in men are high in Western Europe (13.6/100,000), Southern Europe (10.9/100,000), South Asia (12.7/100,000), Southern Africa (11.1/100,000), and Australia/New Zealand (10.2/100,000). In females, incidence is relatively high in Southern Asia (8.3/100,000) (Csikar, *et al*. 2013). Oral cavity cancer is also more common in developing countries with estimated cases of 130,933 in men (3.1% of all cancers in men) and 68,617 in women (1.8% of all cancers in women). It represents the seventh most common malignancy for men in developing countries with an estimated mortality rate of 2.5% (IARC, 2012). The incidence of oral cancer in all age groups has been rising worldwide (Boyle, *et al*. 2008). The similar trend has also been observed in the USA, especially among African American men and in young adults, a group with less tobacco and/or alcohol exposure (Canto and Devesa, 2012).

Oral cancer surveys in Sudan found that squamous cell carcinoma was the commonest malignant lesion representing 66.5% (Ahmed, 2013). 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 (Globalcan, 2012). However IRAC 2012 reported that incidences rate of oral cancer in Sudan are 3.8% for men and 2.3% for women with prevalence are 3.2%. This is strongly attributed to the use of local type of snuff known as Toombak, a very popular material in the Sudanese community (IRAC, 2012; Idris*, et al.* 1998; Idris*, et al.* 1999).

2.4.2. Etiology and risk factors of oral cancer

It has been estimated that the use of tobacco and alcohol accounts for up to 80% of cases of oral cancer. Both act throughout the upper aerodigestive tract, contributing

to the field cancerization effect, and both can induce genetic alterations, such as mutations in the p53 tumor suppressor gene. Other risk factors include viral infection particularly Epstein-Barr virus (EBV) and human papillomavirus (HPV), occupational exposure, radiation, dietary factors, and genetic susceptibility (Sankaranarayanan, *et al*. 1998).

2.4.2.1. Tobacco Smoking

Tobacco exposure is clearly a major risk factor for oral cancer in adults. Tobacco contains at least 55 known carcinogens, which can be grouped into three classes: polycyclic aromatic hydrocarbons, N-nitrosamines, and Asz-arenes (Hecht, 1999). The risk of oral cancer is definitely increased in smokers of all tobacco products. Smoked, chewed, or taken as snuff, although the risk is reported to be higher in smokers who consume cigarettes without filters. Furthermore, there is a strong association of oral cancer and unfiltered tobacco products, namely pipes or cigars (Franceschi, *et al*. 1990; Zheng, *et al*. 2004). The risk of oral cancer increases with amount and duration of smoking, with duration of smoking having a greater impact on risk than amount (IARC, 2004).

2.4.2.2. Smokeless Tobacco

Smokeless tobacco, also known as spit tobacco, has been implicated as a risk factor for oral cancer and is speculated to be a contributing factor for the increase in oral tongue cancer incidence rates among young men in the USA, although there is little US-based data to substantiate the claim. The relationship between the use of smokeless tobacco products and oral cancer is also complicated by significant variations in smokeless tobacco products by region, culture, and time period. However, in a study conducted among women in North Carolina who used predominantly fire-cured dry snuff, a strong dose response relationship was

observed between duration of smokeless tobacco use and risk of buccal and gingival cancer among nonsmokers, with an OR 47.5 (95% CI, 9.1–249.5) for 50 years or longer of use (Winn, *et al.* 2001).

2.4.2.3. Alcohol Use and Tobacco Smoking

The risk of developing oral cancer doubled in people who use both tobacco and alcohol because of a synergistic effect from combined exposure to both products. Attributable risk estimates indicate that tobacco smoking and alcohol account for approximately three-fourths of all oral and pharyngeal cancers in the USA. There is a strong association between the risk of oral cancer and the amount of alcohol consumed and the length of habitual consumption of alcohol and tobacco. The risk may increase directly with alcohol concentration, even after adjustment for total alcohol consumed. It has been demonstrated that the combined use of alcohol and tobacco increased the risk above that expected with either exposure alone (Castellsague, *et al*. 2004).

2.4.2.4. Betel Quid Chewing

Betel quid chewing has long been identified as a major risk factor for oral cancer. It is commonly consumed among older Asians, especially in India. Fifty percent of oral cancers in India occur in the buccal mucosa in contrast to less than 5% in many Western countries (Thomas and Wilson, 1993).

2.4.2.5. Oral Hygiene

Poor oral health such as chronic mucosal irritation or chronic inflammatory state (gingivitis and periodontitis), dental caries, tooth loss (a surrogate for poor oral hygiene), and tartar has been linked to an increased risk for oral cancer. Although both smoking and alcohol consumption have a significant impact on oral health and hygiene, poor oral hygiene may increase the risk of oral cancer by two to four-fold

after adjustment for gender, age, diet, alcohol, and tobacco use. Furthermore, an independent role for oral hygiene was supported by significant elevations in oral cancer risk among nonsmoker nondrinker, suggesting that poor oral hygiene may be an independent risk factor for oral cancer (Talamini*, et al*. 2000).

2.4.2.6. Diet

There is strong evidence in the literature to demonstrate the relationship between diet and risk of oral cancer. Case controlled studies have consistently shown an inverse association between the risk of oral cancer and consumption of fruits and vegetables after adjusting for smoking and alcohol intake (Marshall and Boyle 1996). Frequent consumption of vegetables, citrus fruits, fish, and vegetable oil are the major features of a low-risk diet for cancer of the oral cavity. Patients with the highest quartile of intake of fruits and vegetables had signifi cantly lower risk for oral cancer (OR 0.4; 95% CI, 0.4–0.8) than those in the lowest quartile of intakes as shown in the IARC multinational case – control study (Kreimer, *et al.* 2006)

2.4.2.7. Genetic and Familial Factors

There are indications that there is at least a contributing component related to a genetic susceptibility of the individual exposed to carcinogens and a potential for malignant transformation of the oral tissues. In general, the risk of all oral Squamous Cell Carcinoma (OSCCs) is increased by two to four-fold among individuals with a positive family history (defined as one or more first-degree relatives with the disease), after adjusting for age, sex, alcohol, and tobacco exposure of the index case. The risk is greater (~8–14-fold) if the affected family member is a sibling (Foulkes, *et al*. 1996).

2.4.2.8. Human Papilloma virus

2.4.2.8.1. The HPV Structure and Genome

Human Papilloma virusis 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 5.2×10^6 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, 2009). 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, E4, 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. The E6 and E7 oncoprotiens are usually under control of E1 and E2 inhibitory genes. The potentially oncogenic HPV is divided into high and low-risk types. The high-risk HPV such as 16, 18, 31, 33, 35, 52, 58, 59, 68, 73, and 82 are responsible for malignancies while the low-risk sub types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81) are rarely found in carcinoma and frequently connected with benign and potentially malignant lesions of the oral cancer (Zur Hausen, 1996).

2.4.2.8.2. HPV Oncogenes and their interaction

E1 Oncoprotein

The E1 Open Reading Frames (ORF) is the largest and most highly conserved of all HPV ORFs and codes for a polycistronic RNA, which is translated into 68-85 kDa proteins with both ATPase and helicase activities (Hughes and Romanes, 1993). The E1protein is expressed at low levels in HPV positive cells and has site-specific DNA binding sequences that bind (Longworth and Laimins, 2004, Zur Hausen, 1996). DNA binding is stabilized by complex formation with the E2 protein (Longworth and Laimins, 2004). E1 forms hexamers with high binding affinity for DNA (Wilson, *el al*. 2002). E1 protein is a site-specific DNA-binding protein that possesses ATPase activity (Hughes and Romanes, 1993).

E2 Oncoprotein

The E2 ORF codes for 2-3 proteins all acting as transcription factors (Zur Hausen, 1996). These proteins have a DNA binding region in their C-terminal and regulate viral transcription and replication by forming dimers at specific binding sites. The E2 protein is essential for viral replication, since it directs El to its DNA binding sites and enhances the binding affinity of El to DNA (Kim, *et al*. 2003).

E4 Oncoprotein

The E4 ORF is expressed in low amounts early in the viral life cycle. It is translated from spliced transcripts together with the five first amino acids of E1, where the El sequence is used for initiation of translation. E4 proteins are exclusively found in the differentiating layer of the infected epithelium (Christy, *et al*. 2006). High risk HPV E4 is suggested to be involved in facilitating release of viral particles, since E4 interacts with the keratin networks and causes their collapse. Furthermore, E4 may play a

role in regulating gene expression and has been shown to induce G2 arrest in a variety of cell types (Longworth and Laimins, 2004).

E5 Oncoprotein

The E5 ORF codes for a small highly hydrophobic membrane bound protein, which is primarily expressed late in the viral life cycle in differentiated epithelial cells. The E5 protein of high risk HPVs has weak transforming activities (Longworth and Laimins, 2004), while the corresponding E5 protein in bovine papillomavirus (BPV) is the major transforming protein. BPV E5 and HPV E5 do not share sequence homology and while BPV E5 acts through interactions with the platelet-derived growth factor (PDGF) receptor the HPV E5 is proposed to interact with the epidermal growth factor (EGF) receptor. In HPV E5 over expressing cells/ increased numbers as well as increased phosphorylation of EGF receptors are seen. This is due to impaired degradation of receptors and to recycling of the receptors to the cell membrane (Christy, *et al*. 2006).

E6 Oncoprotein

The E6 oncoprotein of high-risk plays a role in the cellular transformation process. E6 oncoproteins enter the nucleus of host cells via multiple pathways (Le Roux and Moroianu, 2003). Efficient immortalization of keratinocytes requires the combination of E6 and E7 (Munger, *et al.*1998). E6 proteins exert their functions by interacting with cellular proteins. High-risk HPV encoded E6 protein that forms a complex with p53 leading to functional inactivation (Werness*, et al*.1990; Alder, *et al*.1986). High-risk HPV E6 proteins have anti-apoptotic activities and can interfere with the anti proliferative functions of p53. HPV depends on the cellular DNA synthesis machinery and must stimulate S-phase progression to replicate their genome, resulted in P53 over expression which represents a major impairment for viral

replication. High-risk HPV expressing cells have lower p53 levels compared with normal uninfected primary cells (Hubbert*, et al.* 1992). High-risk HPV E6 proteins induce rapid degradation of p53 through ubiquitin-dependent proteolysis (Scheffner, *et al*.1990). This results in bypassing the normal growth arrest signals at the G1/S and G2/M checkpoints leading to accumulation of mutations and later transformation (Fehrmann and Laimins, 2003). E6 proteins of low-risk HPVs did not affect p53 stability or inactivate it (Scheffner, *et al*. 1990).

E7 Oncoprotein

HPV E7 protein binding and degradation of the retinoblastoma protein (Rb) are necessary for its transforming activity. High-risk HPV E7 proteins interact with pRb and induce its proteolytic degradation. The E7 protein together with E6 provide the major transforming activities of HPVs. Expression of the E7 protein in the absence of other viral gene products leads to the transformation of rodent fibroblasts. While E7 alone can immortalize human keratinocytes, the presence of E6 greatly enhances the frequency at which this can occur (Hubbert, *et al*.1999). HPV-16 E7 forms complexes with hypophosphorylated Rb, leading to its inactivation and permits S phase entry (Cobrinik, *et al.* 1992). HPV E7 abrogates Rb mediated regulation of the S transition of the cell division cycle. Rb binds E2F-1 and actively represses transcription from promoters containing E2F-1 sites. Upon phosphorylation late in G1, the hyperphosphorylated Rb does no longer interact with E2F-1, converting E2F-1 into a transcriptional activator. High-risk HPV E7 proteins can interact with Rb and induce its proteolytic degradation, decreasing the abundance of growth suppressive hypophosphorylated pRb increasing the pool of transcriptionally active E2F-1 (Zur Hausen, 1996). Destabilization of the Rb tumor suppressor and stabilization of p53 contribute to HPV16 E7 induced apoptosis and the ability of E7

to induce cellular transformation (Gonzalez, *et al.* 2001). Binding of E7 to hypophosphorylated Rb prevents it from binding to E2F-1 and thereby promoting cell cycle progression allowing for productive replication of HPV genes. HPV-16 E7 plays a major role in inducing centrosome related mitotic disturbances. Abnormal centrosome duplication induced by HPV E7 rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell (Duensing, *et al*. 2001). This activity is, therefore, likely to be functionally relevant to the contribution of high-risk HPVs to malignant progression. E7 of HPV 38 was shown to have in vitro transforming activities (Calderia, *et al*. 2004).

L1 Oncoprotein

The LI ORF is highly conserved between different HPV types and is only expressed in terminally differentiated epithelial cells. It codes for the major capsid protein, which is present in 360 copies per virion. The LI protein self assembles into pentamers, which are the building blocks of the viral capsid and they can also self-assemble into virus-like particles (VLP), but LI does not bind DNA and therefore LI VLPs are generally devoid of DNA (Zhou, *et al.* 1999).

L2 Oncoprotein

The L2 ORF codes for the minor capsid protein, present in around 12 copies per virion. Expression of L2 protein is restricted to terminally differentiated cells of the epithelium. The L2 protein is highly phosphorylated and suggested to be required for encapsidation of viral DNA into the capsid (Zhao, *et al*. 1999), and proposed to act through relocalization of the LI protein to subnuclear domains called promyelocytic oncogenic domains (PODs) (Day, *et al.* 1998).

2.4.2.8.3. HPV Life Cycle and Transmission

HPVs induce hyper prolifarative lesions of cutaneous and mucosal epithelium (Fehrmann and Laimins, 2003). The productive life cycle of HPV is directly linked to the epithelial cell differentiation. Infection by Human papilloma viruses is believed to occur through micro traumas in the epithelium, exposing the basal cells to enter by viruses (Howley and Lowy, 2001). Following entry into keratinocytes in the basal layer, HPV replicates as the basal cells differentiate and progress to the surface of the epithelium (Fehrmann and Laimins, 2003). In the basal layers, viral replication is considered to be non productive and the virus establishes itself as a low-copy-number episome by using the host DNA replication machinery to synthesize its DNA on average once per cell cycle (Flores, 1997). In the differentiated keratinocytes of the suprabasal layers of the epithelium, the virus switches to a rolling-cycle mode of DNA replication, amplifies its DNA to high copy number, synthesizes capsid proteins and causes viral assembly to occur (Flores, 1999). Since HPVs encode only 8 to 10 proteins, they must utilize host cell factors to regulate viral transcription and replication. The E6 and E7 genes encode viral oncoproteins that target p53 and Rb respectively during the viral life cycle; these proteins facilitate stable maintenance of episomes and stimulate differentiated cells to reenter the S phase. The E1and E2 proteins act as origin recognition factors as well as regulators of early viral transcription. The functions of the E5 and E1-E4 proteins are still unknown but these proteins have been implicated in modulating late viral functions. The L1and L2 proteins form capsids for progeny virion generation. The characterization of the cellular targets of these viral proteins and the mechanisms regulating the differentiation-dependent viral life cycle remain active areas for study of these important human pathogens (Longworth and Laimins, 2004).

2.4.2.8.4. Mechanisms of HPV Mediated Carcinogenesis

The influence of viruses and tumor suppressor gene inactivation are of major importance oral cancer. There are reports indicating that HPV and p53 protein alterations frequently coexist in oral lesions and suggest that p53 mutation may be an early genetic event in oral carcinogenesis. Moreover, this coexistence reveals that other environmental carcinogens have a more prominent role in oral carcinogenesis, one that overrides the action of HPV (Aggelopoulou, *et al*. 1998). On the contrary, some reports suggest a stronger association between HPV infection and activation of the H-ras gene in oral verrucous carcinomas. These results continue to to confirm the multihit hypothesis of tumorgenesis and suggest that in some cases of oral cancer at least two of these events are H-ras gene mutation and HPV infection (Anderson*, et al*. 1994).

In this retrospective study of 200 Sudanese patients with oral lesions were screened by molecular methods (PCR) for the presence of HR-HPV subtypes Out of the 200 patients, 12/200 (6%) were found with HR-HPV infection. Of the 12 positive patients, 8/12 (66.7%) were among cases and the remaining 4/12 (33.3%) were among control group. The distribution of different genotypes was: type HPV 16 6/12 (50%), HPV18 4/12 (34%), HPV 31 1/12 (8%) and HPV 33 1/12 (8%). In view of these findings, HPV particularly subtypes 16 and 18 play a role in the etiology of oral cancer in the Sudan (Babiker, *et al*. 2013).

In Sudan 2012, Ahmed and Abusail are determine the frequency and genotype of human papillomavirus (HPV) infections in head and neck squamous cell carcinomas (HNSCCs) and benign head and neck tumours. Six of the 150 (4%) HNSCCs were HPV positive. HPV16 was the most prevalent type, with single infections present in 3/6 (50%) cases, whereas HPV18 and HPV33 were detected in 2/6 (33%) and 1/6

(17%), respectively. HPV infections were detected in 3 (50%) cases of oral cavity and 3 (50%) cases of pharynx. There was a significant association between HPV infection and HNSCCs ($P < 0.05$). The present data support the importance of HPV infection in oral and larynx tumours (Ahmed, *et al*. 2013).

In 2010, Ahmed and Eltoom are detected the role of high risk Human Papilloma viruses (HPV) 16 and 18 in oral squamous cellcarcinomas (OSCC), 40 SCCs and 15 benign lesions were analyzed for the presence of HPV DNA by polymerase chain reaction (PCR). Pearson Chi-square test for statistical significance (P value) with the 95% confidence level and confidence intervals wereused. HPVDNA was detected in 15% of cases (six out of 40 cases), and none of controls (n=15), P <0.0001. Among thesix positive cases four were HPV type 18 and the remaining two were type 16.These results provide evidence supporting causal association between HPV infection and oral SCC in Sudan (Ahmed and Eltoom, 2010).

2.4.2.8.5. Detection techniques of HPV in oral cancer

Cytological and Histopathological Examination

The detection of HPV in the oral mucosa may be done by cytology and histological examination. On the basis of cytology and histopathology, HPV infection is characterized by koilocytosis, perinuclear cytoplasmic haloes, nuclear dysplasia, atypical immature metaplasia and binucleation. These methods show limited sensitivity and are unable to determine which types of HPV are involved in the infection of the epithelial cells suggested that oral scrapes or rinse samples, with their greater surface area of mucosa than with biopsies, are less invasive. High risk HPV detection in oral exfoliated cells is a reliable biomarker of an HPV-related head and neck cancer risk. A drawback is that not all patients who have HR-HPV types in

oral exfoliated cells are detected with HPV DNA in the primary tumor (Smith, *et al*. 2004).

Immunohistochemical staining

Immunohistochemical staining has revealed the presence of HPV capsid antigens in HPV-infected cells. Capsid antigens, however, has rarely been detected in high-grade neoplasias or invasive cancer, probably because such tissue contains limited numbers of highly differentiated squamous epithelial cells. Consequently, the majority of oral lesions that contained HPV structural antigens were either benign or precancerous. Inconsistencies in antigen detection also resulted from sampling error. Variable expression or lack of HPV capsid antigens, destruction of antigens during cellular processing or long term storage, or lack of sensitivity to a particular assay (Syrjanen, 1990).

Molecular methods: Polymerase chain reaction (PCR)

This method is used to detect HPV in Oral cancer, and is based on a polymerase chain reaction (PCR) and uses either HPV type–specific DNA primers (DNA oligomers that detect only HPV-16) or consensus primers (detect shared sequences across multiple types of HPV) (Kay, *et al*. 2002). It allows for amplification of DNA isolated from tumor cells. Briefly, a DNA polymerase recognizes an oligonucleotide primer bound to a specific DNA sequence. By using two primers that flank a targeted region of interest, after several round of amplification, the target has been amplified sufficiently to allow for visualization on an agarose gel. Either degenerate primers which amplify DNA sequences from multiple subtypes of HPV, or specific primers which amplify DNA sequences from a single subtype of HPV can be used so that PCR can be used both as a screening test for any HPV infection and to confirm the subtype of HPV identified. The resulting PCR products from one or more HPV

subtype can be detected by oligonucleotide array. Briefly, type-specific probes are plated onto an array. The PCR product is hybridized to the chip and resulting signals are visualized with a DNA chip scanner. This type of assay can have a sensitivity approached 95% and has the added benefit of being able to detect multiple HPV types within a single specimen (Kim, *et al.* 2003; Seo, *et al*. 2006). This type of assay can also be used to detect amplified mRNA sequences that may correlate with progression to invasive disease (Szarewski, *et al.* 2008). Unlike standard PCR that amplifies genomic DNA, reverse transcriptase PCR (RT-PCR) utilizes RNA that is first reverse transcribed into cDNA. Following the generation of cDNA, PCR is performed as described above. Quantitation of products for both standard PCR and RT-PCR can be performed by agarose gel electrophoresis. Alternatively, products can be detected in real-time with the use of DNA sequence-specific probes or fluorescent dyes. QRT-PCR allows for relative quantitation of RNA levels if appropriate controls are performed and can be used to determine whether an HPV infection is transcriptionally active (*i.e.*, does the viral DNA present result in production of mRNA and viral proteins). Due to the high dynamic range of qRT-PCR (>7 logs of input), one can detect RNA sequences present at very low concentrations that may not be identified by conventional PCR (Heid, *et al*. 1996). Both PCR and RT-PCR are highly sensitive tests owing to the exponential amplification of target sequences that lie between two priming sequences. In theory these techniques can detect a single copy of a target sequence within a given sample. In reality, this high sensitivity can lead to false positive results either through the inclusion of random HPV genomes (particularly troublesome in labs which commonly study HPV) or by detection of HPV genomes within the investigated tissues, but that are not causative for the malignancy. Probe based qRT-PCR, which

utilizes a third probe that lies within the amplified region can significantly decrease the risk of false positives. These tests can also be used to estimate the integration status of the HPV genome. Upon integration of the viral genome, both the L1 and E2 genes are typically disrupted and lost. Samples that retain E6 and E7 expression, but do not express L1 or E2 are considered to harbor integrated HPV (Liu, *et al*. 2010; Huang, *et al*. 2008). While those that express all 4 viral genes harbor episomal HPV. QRT-PCR methods can be automated at each step from purification of cellular RNA to production of cDNA to amplification, detection, and analysis so that little user effort is required. However, for both tests, the possibility of target degradation when fresh frozen tissue is unavailable represents a significant limitation in most clinical settings.

DNA in situ hybridization (ISH)

DNA in situ hybridization (ISH) does not only detect the viral DNA, but can also localize the virus within in the natural morphology of the tissue. Tissue sections are put on slides and the tissue sections are hybridized with labeled DNA or RNA probes after denaturation. The sensitivity differs between different ISH methods, but can identify approximately down to 20-25 HPV copies/ cell. ISH can also be used to see if the viral DNA is integrated into the chromosome or is episomal in the nuclei (Zumbach*, et al.* 2000).

Southern blot hybridization

It has long been used to identify HPV DNA. Purified DNA is enzymes and then separated by agars gel electrophoreses. After denaturation the DNA is transferred to a membrane and HPV specific sequences are identified through hybridization (specific complementary nucleotide binding) to labeled cloned HPV DNA. The sensitivity is about 0.1 HPV genome copy per cell. Furthermore, the method can

give information regarding if the virus DNA is integrated or episomal. The main advantage of this technique is the high specificity in HPV typing, and thedisadvantage is that the technique is time consuming and a relatively large amount of DNA is needed (10 ug) (Syrjanen, 1990).

Dot blot hybridization

Dot blot hybridization is a method where extracted DNA, without restriction enzyme digestion, is transferred and bound to a membrane in its single-stranded form. Similar to Southern blotting, HPV specific sequences are identified by hybridization with labeled cloned HPV DNA. The sensitivity is about 1 genome copy per cell, using 300-500 ng sample DNA. One advantage of this method is that it is relatively fast, however, its specificity is lower than that of the Southern (Syrjanen, 1990). A variant of the assay is to bind HPV PCR products to the membrane, instead of extracted DNA. Reverse dot blot is another variant for HPV typing, where different types of known HPV DNA are fixed on the membrane and labeled extracted DNA or PCR products are used as probes (de Villiers, 1992).

Gene Expression: DNA Microarray

DNA microarray is a compilation of microscopic DNA spots on a solid surface by covalent attachment to a chemical matrix. Each gene on the solid supports referred to as spot or probe is usually less than 200 μm in diameter. Each spot has a unique sequence different from the others in the array and will hybridize only to its complimentary strand. This technique uses a DNA probe labeled with either a radioisotope or a fluorescent tag. The probe is applied to the fragment of DNA or RNA to be studied and by the rules of base pairing (A to T, C to G) "sticks" to its complementary sequence. This technology has made it possible to miniaturize

methods of probe detection for DNA and allow detection of several thousand DNA or RNA sequences in one experiment (Vijver, *et al*. 2002).

Hybrid capture II (HC II)

The HC II technique is a nucleic acid hybridization assay with signal amplification that utilizes microplate chemiluminescent detection. First, double stranded DNA is denatured by using a strong alkaline denaturation solution that converts it into single stranded DNA (ssDNA). This ssDNA is then hybridized in-solution to a cocktail of specific 13 high risks HPV RNA probes. The resultant DNA-RNA hybrids are captured onto the surface of a micro-well plate coated with particular antibodies for DNA-RNA hybrids. The immobilized hybrids are reacted with alkaline phosphate conjugated antibody and detected by cleavage of the chemiluminescent substrate. The emitted light is measured as relative light unit (RLU) in a luminometer. The intensity of the light is proportional to the amount of target DNA in the sample. Specimens with RLU greater than or equal to the mean of the three positive control values are considered HPV positive (Braakhuis, *et al.* 2004).

2.5. Diagnosis of oral cancer

2.5.1. Initial evaluation

The initial assessment of the primary tumor is based upon a combination of inspection, palpation, indirect mirror examination, and direct endoscopy. Physical examination should include careful assessment of the nasal cavity and oral cavity with visual examination and palpation of mucous membranes, the floor of the mouth, the anterior two-thirds of the tongue, palate, tonsillar fossae, buccal and gingival mucosa, and posterior pharyngeal wall (Rennemo, *et al*. 2011; Strobel, *et al*. 2009; Hujala, *et al*. 2005).

2.5.2. Imaging studies

These include: (CT scan), magnetic resonance imaging (MRI), positron emission tomography (PET), and integrated (PET/CT) are important for assessing the degree of local infiltration, involvement of regional lymph nodes, and presence of distant metastases or second primary tumors (Rasch, *et al*. 1997).

2.5.3. Magnetic resonance imaging (MRI)

MRI provides superior soft tissue definition compared with CT (Sakata, *et al*. 1999), and can often provide information that is complementary to CT. For example, MRI can provide more accurate definition of tumors of the tongue and is more sensitive for superficial tumors. MRI is also better than CT for discriminating tumor from mucus and in detecting bone marrow invasion. For this reason, MRI can be useful for evaluation of cartilage invasion, particularly for non-ossified cartilage that can pose difficulty for CT. On the other hand, CT scanning is better than MRI for detection of bone cortex invasion since MRI shows no bony detail.MRI is superior to CT for evaluation of perineural spread, skull base invasion, and intracranial extension of oral cancer (Rasch, *et al*. 1997).

2.5.4. Oral brush biopsy

Oral cells can be obtained by different physical systems of scraping the surface of the mucosa, by rinsing the oral cavity or even by taking a sample of saliva from the patients. The reliability of the different instruments used in oral exfoliative cytology has been reviewed in different studies. The ideal instrument used for making a good cytological smear should be easy to use in any location, cause minimum trauma and provide an adequate and representative number of epithelial cells. It has been shown that a brush is an adequate instrument due to its ease in sampling and to the quality of the oral cytologic sample Brush biopsy is a simple, relatively inexpensive,

high sensitive, risk-free method of screening for cancer and serves as an aid to the clinical examination (jones, *et al.* 2008; Brocklehurst, *et al*. 2013).

2.5.5. Exfoliative cytology

Exfoliative cytology is a simple non-aggressive technique that is well accepted by the patient, and that is therefore an attractive option for the early diagnosis of oral cancer, including epithelial atypias and especially squamous cell carcinoma. However, traditional exfoliative cytology methods show low sensitivity in the diagnosis of these pathologies. This low sensitivity is attributable to various factors, including inadequate sampling, procedural errors, and the need for subjective interpretation of the findings. (Diniz, *et al.* 2004).

2.5.6. Cytomorphometry

It is 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 (Ogden, *et al.*1997; Lynch, 1984).

2.5.7. Histopathology

Oral squamous cell cancer arises from normal surface epithelium. The surface epithelial cells undergo gradual changes from clinically undetectable premalignant lesion to clinically identifiable premalignant lesion. These premalignant stages are often reversible and are readily curable. Symptoms of premalignant conditions can be identified by screening alone; however most often these remain unnoticed. Patients report only after the disease advances to an irreversible malignant lesion (Taylor, 1993). Oral pre-cancerous lesion has been defined by an International Working Group as 'morphologically altered tissue', which in cancer is more likely to occur than in its apparently normal counterpart. (Axell, *et al.*1991) There are two

major clinically visible pre-malignant lesions namely leukoplakia and erythroplakia. Dysplastic lesions are more likely to undergo malignant change since chances of malignant transformation increase with increase in the severity of dysplasia (Johnson, *et.al.* 2007). The main morphological features of dysplasia are hyperchromatism and loss of polarity of basal cells. Increased nuclear to cytoplasmic ratio is often characteristic of dysplastic lesions. Carcinoma in situ has the highest risk among the histologically identifiable pre-cancerous lesions, showing marked epithelial dysplasia involvingfull thickness of the epithelium. Since carcinoma in situ is a stage that appearsbriefly and quickly progresses on to invasive lesion, it is not generally reported (Smith, *et.al.*2004). High-risk squamous cell carcinomas are those that are associated with short survival time. This is usually due to early recurrence of neoplasm after thetreatment. Despite identical staging, prediction of prognosis based on histopathology alone has not proven useful in oral cancer. Within the individual oral cancer there is often considerable variation in histological features in different parts. Histopathology reports based on structural criteria is suggestive of malignancy rather than the functional activity of the neoplastic cells (Johnson, *et.al.* 2007). Broder's classification based on degree of differentiation is the most commonly followed pathological grading system. Percentage of differentiated cells is used to gradethe tumor as well differentiated, moderately differentiated, poorly differentiated and undifferentiated tumors (Pindborg, *et.al*. 1997). Further modification of this grading was done by including additional information such asstructural cohesiveness of cells, tendency to keratinize, nuclear abemtions, and number of mitoses above the basal layer (Anneroth, *et.al*. 1996).

Staging of oral cancer:

The tumor node metastases (TNM) staging system of the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) is used to classify cancers of the oral cancer. The T classifications indicate the extent of the primary tumor and are site specific; there is considerable overlap in the cervical node (N) classifications. Oral cancer are staged accordingto size and site of the primary tumor (T), number and size of metastases to the cervical lymph nodes (N), and evidence of distant metastases (M). Staging usually requires imaging with CT, MRI, or both, and often PET (Edge, *et al.* 2010).

Table (1.2) Staging of oral Cancer

Stage	T (Size of Primary Tumor)	N (Lymph Node Involvement)	M (Distant Metastases)
I	T1: tumor < 2 cm	N0: no nodal involvement	M0: no metastases
II	T2: tumor > 2 and < 4 cm	N1: ipsilateral lymph node involvement < 3 cm	MI: distant
III	T3: tumor > 4 and < 6 cm	N2: ipsilateral lymph node involvement > 3 cm	metastases
	T4: tumor > 6 cm	or multiple ipsilateral nodes	
IVA	T1–T4a: no local extension	N2a: single ipsilateral node > 3 and < 6 cm	
IVB	T1–T4b: local extension (clinical or	N2b: multiple ipsilateral nodes < 6 cm	
	macroscopic extension to skin, bone,	N2c: bilateral or contralateral nodes < 6 cm	
	nerve)	N3: lymph > 6 cm	

2.5.8. Molecular methods

Analyzing differences in gene expression patterns across individual patients with a certain type of cancer may reveal molecular differences that permit refinements in their classification, prognostication, and treatment selection. At least some data suggests that primary oral cancer may carry specific molecular changes that are capable of predicting the presence of (or potential for) cervical nodal metastases. However, these approaches remain investigational (Roepman, *et al.* 2005; Kleer, *et al.* 2006).The genetic alterations observed in oral cancer include activation of proto-

oncogenessuch as cyclin D1, MYC, RAS, EGFR and inactivation of tumor suppressor genes such as those encoding $p16^{INK4A}$ and p53 and other putative suppressor loci. Early changes include loss of tumor suppressor genes on chromosomes 13p and 9p, followed by 17p. p53 mutations and overexpression are seen in the progression of preinvasive lesions to invasive lesions. p53 mutations are more frequently reported in developed (40-50%) than in developing countries (5-25%). Tumors from India and South East Asia are characterized by the involvement of RAS oncogenes, including mutation, loss of heterozygosity (HRAS) and amplification (KRAS and NRAS). Various genetic polymorphisms in genes such as GSTM1 or CYP450A1 are associated with oral carcinogenesis (Jonah, *et al.* 2010).

2.5.9. Immunohistochemistry

Immunohistochemistry has greatly assisted in the identification of tumors that cannot be accurately identified using routine histopathological procedures (Ramaekers, 1992).In some undifferentiated tumors, subtle features of epithelial versus mesenchymal differentiation can often be appreciated, which assist the immunohistochemical approach to these tumors. Some tumors, however, may not fit into either of these two categories because of their overlapping histological features. Nevertheless, making the correct histopathological diagnosis is essential in deciding the appropriate therapy.The immunohistochemical evaluation of undifferentiated tumors should first aim at a broad lineage determination of the neoplasia. Based on the result of the screening panel, a more detailed or specific panel should then be applied to further sub classify the tumor or to confirm a particular diagnosis (Bahrami, *et al*. 2008).

2.5.9.1. Tumor suppressor genes

2.5.9.1.1. The P53

P53 is a tumor suppressor gene, located on chromosome 17p13.1, which plays a role in cellcycle progression, cellular differentiation, DNA repair, and apoptosis. A major function of p53 is to serve as a guardian of the genome. p53 is the most commonly mutated gene and is altered in over 50% of all cancers, including 25–70% of oral cancers (Levine, 1997).The p53 protein is involved in the maintenance of the cellular integrity after DNA damage by transiently blocking the cell cycle progression, direct or indirect stimulation of DNA repair mechanism and triggering of apoptosis if DNA repair fails. Alterations in p53 through the loss of heterozygosity, point mutations, deletions, insertions or interaction with viral proteins marking early event of oral cancer carcinogenesis can be detected as early as pre- malignant lesions. P53 mutations are present before and maintained through the metastasis (Joerger and Fersht, 2008). Mutations in p53 occurring in mechanisms of gene mutation and viral interaction which might affect its function include loss of a wild-type activity, a dominant-negative effect or gain of oncogenic potential (Ko*, et al*. 1996; Aid, *et al*. 2013). The tumor suppressor p53 which codes for 11 exons is active in tetrameric form with four identical chains of 393 residues. The structure of the gene has been broken down. P53 comprises of an N-terminal transactivation domain (TAD), followed by a proline-rich region (PRR), the central DNA-binding domain (p53C), the tetramerization domain (TET), and the extreme C terminus (CT). The DNA binding domain is the domain where most cancer-associated p53 mutations are located. A compilation of 84 studies in which IHC and sequencing experiments were performed on the same tumor sets revealed that sensitivity of IHC

for mutant p53 was 75% and the positive predictive value was 63% (Greenblatt, *et al.* 1994).

2.5.9.1.2. The Rb protein

Is a tumor suppressor protein that is dysfunctional in several major cancers. One function of pRb is to prevent excessivecell growth by inhibiting cell cycle progression until a cell is ready to divide. It is also a recruiter of several chromatin remodeling enzymes such as methylases and acetylases (Murphree and Benedict, 1984). The Retinoblastoma protein (pRb) is involved in regulating a variety of cellular functions including cellular division, differentiation, senescence and apoptosis. pRb arrests cells during the G1 phase of the cell cycle by repressing transcription of genes required for G1 to S transition (Harbour and Dean, 2000). A major cellular target of pRb is the E2F family of transcription factors that binds to the promoters of many genes that are involved in cell cycle progression like cyclin E. The Rb pathway is thought to be virtually disrupted in all cancers (Jayasurya, *et al.* 2001). Several human tumors show mutations and deletions of the Rb gene (13q14), with inherited allelic loss of Rb conferring increased susceptibility to cancer formation. Previous immunohistochemical studies from our lab have demonstrated an overexpression of the Rb gene in more than 62% of the cases (Hatakeyama and Weinberg, 1995).

2.6. Management of oral cancer

Prevention

Avoidance of recognised risk factors: Not smoking or chewing tobacco. Limiting alcohol consumption. Increasing the intake of fruit and vegetables (especially tomatoes), olive oil and fish oils. Reducing the intake of red meat, fried food and fat, are the most effective form of prevention. Regular dental examinations may identify

pre-cancerous lesions in the oral cavity. When diagnosed early oral cancer can be treated more easily and the chances of survival increase tremendously. It is expected that HPV vaccines may reduce the risk of HPV induced oral cancer (Smith, *et al.* 2004).

Treatment

The treatment of oral cancers mainly consists of surgical treatment, radiotherapy, chemotherapy and immunotherapy as auxiliary treatments. These treatment methods are used in combination, based on the size of the primary lesion, carcinogenic area, presence or absence of metastasis to the cervical lymph nodes, and histological staging (Woolgar, *et al.* 1995).

Surgery

Surgery; to remove the tumor in the mouth is the usual treatment for patients with oral cancer. If there is evidence that the cancer has spread, the surgeon may also remove lymph nodes in the neck. If the disease has spread to muscles and other tissues in the neck, the operation may be more extensive (Woolgar, *et al.* 1995).

Radiation therapy

It is the most common form of treatment. There are different forms of radiation therapy, including 3D conformal radiation therapy, intensity-modulated radiation therapy, and brachytherapy, which are commonly used in the treatments of oral cancer (Bonner, *et al.* 2006)

Chemotherapy

Chemotherapy is the use of drugs to kill cancer cells. Researchers are looking for effective drugs or drug combinations for oral cancer therapy. They are also exploring ways to combine chemotherapy with other forms of oral cancer treatment to help destroy the tumor and prevent the disease from spreading (Bingham, 1986).

Targeted Therapy

According to the National Cancer Institute, is "a type of treatment that uses drugs or other substances, such as monoclonal antibodies, to identify and attack specific cancer cells without harming normal cells." Some targeted therapy used in squamous cell cancers of the oral include cetuximab, bevacizumab, erlotinib, and reovirus. The best quality data are available for cetuximab since the 2006 publication of a randomized clinical trial comparing radiation treatment plus cetuximab versus radiation treatment alone (Bonner, *et al*. 2006).

2.7. Prognosis of oral cancer

Lymph node involvement and tumor size are the most important prognostic factors. Data for the United States for 1975-2007 reports a year-year survival for all stages of oral cancer of 60.9 percent, 82.5 percent for early stage disease, and 54.7 percent for locally advanced oral cancer (Ries, *et al.* 2008). The reported five-year overall for oral cancer (for all stages combined) from populations in low- and middle-income countries (LMICs)such as China, Cuba, India, Pakistan, and Thailand ranged from 26 percent to 45 percent; for Stages I and II, the survival rates ranged from 36 percent to 83 percent. The inferior survival rates in (LMICs) versus high-income countries (HICs) reflect disparities in the availability, accessibility, and affordability of diagnostic and treatment services (Sankaranarayanan and Swaminathan, 2011).

Chapter Three

Materials and Methods

3.1. Study design

This is a descriptive cross sectional study aimed to identify the association of high risk HPV with immune-expression of mutated p53 and Rb proteins among Sudanese patients with oral lesions. The study was conducted in Khartoum state hospitals (Military hospital, Radiation Isotope centre, and Alribat hospital) during the period from January 2012 to January 2015.

3.2. Materials

Formalin-fixed paraffin wax processed blocks previously taken from patients presented with oral lesions. Data concerning any specimen were taken from histopathology laboratories.

3.3. Sample size

All tissue specimens were taken from three hospitals (200) oral lesions was used in this study. The sample size was calculated using software known as the survey system, available at http://www.surveysystem.com/sscalc.htm. The system inertly relies on the equation: n = z2pq /d2 (n = sample size; z = the standard normal deviate, usually set at 1.96, which corresponds to the level of the 95% confidence level; p = the proportion to the target population. q=1-p, d=degree of accuracy desired, set at 0.05.

3.4. Sample processing

Histopathology was performed using conventional hematoxylin and Eosin Mayer's method. The diagnosis was based on clinical examination and histological features of the biopsy.

3.4.1. Immunohistochemistry

Paraffin embedded blocks of oral cancer tissues as well as benign oral tumors were retrieved from histopathology laboratories and cut into (3 µm thick) sections using rotary microtome. The sections were mounted on poly-L-lysine-coated slides and dried in hot air oven at 60°C for 1 hour. The sections were dewaxed in xylene 5 minutes, three times, and rehydrated through descending grades of ethyl alcohol beginning with 100% ethyl alcohol, then 90% ethanol, 70% ethanol and finally to distilled water, 4 minutes for each change, then the sections were washed 3 times with PBS, three minutes for each. The sections were boiled in the Target Retrieval Solution of Dako (Real Envision Detection Kit) in a water bath at 95°c for 30 min, then left to cool at room temperature and washed three times with PBS. 0.3% hydrogen peroxide in methanol were added to each section for 15 min to block endogenous peroxidase activity, and then washed three times with PBS. The following antibodies (Abs) were used: primary mouse monoclonal mutant Rb antibody and primary mouse monoclonal mutent p53 antibody. (Gene tech company limited, Shanghai, China) at a working dilution of 1/100, at 37°C for 30 min; After two washes in PBS, sections were incubated with ChemMateTM EnVision of+/HRP (Gene tech company limited, Shanghai, China), a secondary antibody at room temperature for 30 min, then washed three times in PBS. The immunoreactivity was detected using diaminobenzidine (DAB) (Gene Tech Company limited, Shanghai, China) in a dilution 1/100 as the final chromogen for

10 min, and then washed in DW for 3 min. Finally, sections were counterstained with Mayer's Hematoxylin for 3 min, and washed in running tap water 5min, then dehydrated through a sequence of increasing concentrations of alcoholic solutions and cleared in xylene then mounted with DPX. During each IHC assay, proof slides were coupled with negative and positive controls provided by the manufacturer for each marker, and reactions were observed appropriately. IHC stained sections were examined under the light microscope (Olympus CHT, Optical.Co.Ltd, Japan) using 4×, 10×, 40× 100×, objective and eyepieces of 10× giving a maximum magnification of 1000. Mutated P53 and Rb were observed only as a nuclear staining of epithelial cells, and the nuclei with clear brown color, were scored as positive. The intensity of immunohistochemical staining for each marker was scored by two investgators based on subjective evaluation of color exhibited (brown) by antigen, antibody and chromogen complex. It was scored as 0 for negative (no color), 1+ for weak (light brown color), 2+ for moderate (dark brown color), and 3+ for strong staining (very dark brown color) with 0 or 1 scores defined as negative and 2 or 3 defined as positive (Pu, *et al*. 2009).

3.4.2. Molecular method

2.4.2.1. DNA extraction

Paraffin embedded blocks of oral cancer tissues as well as benign oral tumors were cut into small slices of sections of thickness 20µ, the tissue sections were placed into 1.5ml centrifuge tubes, then xylene was added to each tube for dewaxation for 30 minutes. The samples were centrifuged at 13000 rpm for 5 minutes and the supernatant was then removed using Pasteur pipette. The sections were then rehydrated through different grades of ethyl alcohol beginning with 100% ethanol,

80%, 60%, 40% and finally placed in deionized water, 10 seconds in each of these rehydrating solutions followed by centrifugation at 13000 rpm for 5 minutes.

Digestion method

To achieve a digestion, 200µl lysates FTL (25-50 mg/ml) and 20µl of protein enzyme K solution (Beijing Aidlab Biotechnology Co., Ltd.). (20mg/ml) were added to the sections into Eppendorf tube, then briefly vortexed and incubated at 37°C in water bath overnight. 10ul proteinase K solution (20mg/ml) was added then incubated in water bath at 55 C for 2 hours. 200ul combination of liquid CB was added and immediately vortexed 20 seconds and placed in water bath at 70 °C for 10 minutes. The samples were allowed to cool then 100ul isopropanol were added and immediately vortexed for 30 seconds then transferred to absorption column (adsorption column into the collection tube) and centrifugedat 13,000 rpm for 60 second, the collection tube waste was discarded. 500ul of in hibitor removal solution the IR were added and centrifuged at 12,000 rpm for 30 seconds, and then the waste was discarded. The samples were washed with 700ul water buffer and centrifuged at 12,000 rpm for 30 seconds then the wastes were discarded, this process repeated two times. The adsorption column AC was removed and the collection tube was centrifuged at 13,000 rpm for 2 minutes, then the water buffer was discarded.100ul of elution buffer were added to the sample for 5 min at RT then centrifuged at 12,000 rpm for 1 min.

3.4.2.2. Polymerase chain reaction (PCR): Genotyping of HPV

Amplification of HPV

12 types' specific primers of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were used to detect high risk HPV in oral tissues (benign as control, cancers as cases). Amplification was performed according to HPV kit from (Sacace

technologies- Casera –Italy). The PCR was carried out in a total reaction volume of 40 µl containing between 20µl mix-1(contained in PCR tubes), 10 µl of mix-2 and 10 µl of extracted DNA (sample). Negative control and positive control 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 (reagents and primers provided by sacace technologies- Casera –Italy). The PCR program was described in Table 1.

Table (3.1) Show PCR Program used for amplification of HPV genes

Steps	Temperature	Time	Cycles
0	95°C	**Pause**	
1	95°C	15 min	1
2	95°C	15 sec	42
	65°C	40 sec	
	72°C	25 sec	
3	72°C	1 min	1
4	4°C	**Storage**	

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).

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 322 bp, HPV 18 should be 457 bp,HPV 33 should be 398 bp and HPV 31 should be 263 bp.

Table (3.2). 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	322
18	CAC TTC ACT GCA AGA CAT AGA	457
31	GAA ATT GCA TGA ACT AAG CTC G	263
33	ACT ATA CAC AAC ATT GAA CTA	398
35	CAA CGA GGT AGA AGA AAG CAT C	358
39	GAC GAC CAC TAC AGC AAA CC	280
45	GTG GAA AAG TGC ATT ACA GG	151
51	GAG TAT AGA CGT TAT AGC AGG	223
52	TAA GGC TGC AGT GTG TGC AG	229
56	GTG TGC AGA GTA TGT TTA TTG	181
58	GTA AAG TGT GCT TAC GAT TGC	274
59	CAA AGG GGA ACT GCA AGA AAG	215

3.5. Statistical analysis

Data obtained from this study were analyzed by using statistical package for social science software (SPSS v.13). A value of 0.05 was considered the value of

statistical significance for all statistical tests in the present study. Chi-square test was used to state the significance of results.

3.6. Ethical consideration

The study proposal was approved by Research Board and the department of histopathology and cytology, Collage of Medical Laboratory Science, Sudan University of Science and Technology and approved from three hospitals.

Chapter Four

Results

In this study, 200 patients with ages ranged from 14 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 oral benign of patients were decreasing with the increase of age, as shown in Figure (4.1).

The male female ratio is 1.38:1.0. The distribution of males and females was relatively similar among different age groups as indicated in table (4.1). The majority of oral lesions were found in Khartoum (58.5%), followed by the West (17.5%), North (13.5%), East (9%) and South (1.5%). as shown in Figure (4.2).

Ninety percent of oral cancer was squamous cell carcinoma (OSCC), 4% were adenocarcinoma, 4% were mucoepidermal carcinoma, and 2% were Rhabdomyo-sarcoma. The main types of benign oral tumors were inflammatory conditions followed by pleomorphic adenoma, squamous papilloma, pyogenic granuloma, reactive hyperplasia, fibroma, haemangioma, ameloblastoma and hyperkeratosis, representing 37/100(37%), 24/100(24%), 8/100(8%), 7/100(7%), 7/100(7%), 5/100(5%), 5/100(5%), 4/100(4%) and 3/100(3%), respectively.

Distribution of the study population by behavior of lesions and gender is shown in Figure (4.3). 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.

According to the lesion sites, 44 cases of oral cancer were found in Buccal mucosa, 21 cases in Salivary gland, 11 cases in Oropharynx, 10 cases in Tongue, 6 cases in Jaw, 5 cases in lip and 3 cases were found in Gingival, representing 44%, 21%, 11%, 10% 6%, 5%, and 3%, respectively. In benign oral tumors, 27 cases were found in Buccal mucosa, 25 cases in Salivary gland, 17 cases in Tongue, 10 cases in Oropharynx, 10 cases inlip, 9 cases in Jaw and 2 cases in Gingival, representing 27%, 25%, 17%, 10%,10%, 9%, and 2% respectively as shown in Figure (4.4).

High frequency of oral lesions was seen among patients with buccal mucosa lesions, constituting 41/71(58%) males and 30/71(42%) females, followed by the salivary glands 24/46(52%) males and 22/46(48%) females, Patients with tongue lesions represented 19/27(70%) were males and 8/27(30%) were females. Oropharynx lesions were found in 16/21(76%) males and 5/21(24%) females. Lip, 6/15(40%) males and 9/15(60%) females. Jaw lesions were found in 5/15(33.3%) males and 10/15(66.7%) females. Gingival lesions were seen in 1/5(20%) males and 4/5(80%) females as show in Figure (4.5).

Distribution of tumors, age, gender, and site of lesions by HPV genotyping were indicated in Table (4.3). HPV genomic materials using E6 and E7 primers were detected in 12/200 (6%) of oral lesions. Of these, 7/12 (58%) HPV-16, 3/12 (25%) HPV-18, 1/12 (8%) HPV-31, and 1/12(8%) HPV-33. Out of the 12 HPV; 10/12(83.3%) HPV were found in malignant lesions as shown in Figure (4.5), whereas, 2/12(16.7%) HPV were found in benign lesions. Consequently, the risk associated with HPV infection was found to be statistically significant (P=0.02).The age group 40-49 years was the most susceptible to HPV infection, followed by 50+ and 30-39 constituting 6/12(50%), 4/12(33%), and 2/12(17%) respectively. The predominant isolated HPV genotypes were HPV16, 7/12(58%), followed by HPV18

3/12(25%), HPV31 1/12(8%) and 1/12(8%) of HPV33 genotypes. Regarding 8/12(67%) of HPV positive were found among males and the remaining 4/12(33%) were found among females. (41%) HPV-16, (17%) HPV-18 and (8%) HPV-33 were detected among males, whereas, (17%) HPV-16, (8%) HPV-18 and (8%) HPV-31 were detected among females as show in table (4.2).

The great majorty of HPV positive lesions were found in tongue (33%) followed by jaw (16%), buccal mucosa (16%), oropharynx (16%), salivary glands (8%) and gingiva (8%). Of the 7(100%) HPV-16 positive cases, (29%) were detected in the buccal mucosa, (43%) in the tongue, (14%) in the oropharynx and the remaining (14%) was detected in the jaw. Of the 3(100%) cases of HPV-18, (33%) were detected in the salivary glands, (33%) in the tongue, and the remaining (34%) was detected in the gingiva, 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 as show in Table (4.2).

Mutations of P53 and Rb were expressed only in malignant oral lesions, 34/100(34%) and 25/100 (25%) respectively. P53 and Rb mutations were not detected in in benign oral lesions. The risk of mutations of both p53 and Rb associated with oral cancer were found to be statistically significant P-value< 0.001 as shown in table (4.4) and Photomicrograph (4.1 and 4.2).

No mutation of were found among those benign lesions with HPV infections (2 cases). However, p53 mutations were found in 3/10(30%) of malignant tumors with HPV infection, as well as 1/10(10%) with Rb gene mutation, P. value=0.497 indicated in table (4.5). In regard to the relation between HPV genotyping and P53, Rb genes mutations, most of the P53 and pRb mutations were found in HPV-16.

Table (4.1) Distribution of the study population by age and gender

Age groups	Gender		Total
	Male	Female	
≤20 years	12	13	25
20-29	14	17	31
30-39	15	16	31
40-49	15	14	29
> 50	56	28	84
Total	122	88	200

Table (4.2): Distribution of tumor, age, sex and site of oral lesion by HPV genotyping.

Variable	Category	HPV genotyping				Total	p.value
		16	18	31	33		
Tumor	*Malignant*	5	3	1	1	10	0.02
	Benign	2	0	0	0	2	
	Total	7	3	1	1	12	
Age	*< 20 years*	0	0	0	0	0	0.72
	21-29	0	0	0	0	0	
	30-39	1	1	0	0	2	
	40-49	3	2	0	1	6	
	50+	3	0	1	0	4	
sex	*Male*	5	2	0	1	8	0.37
	Female	2	1	1	0	4	
Site of lesion	*Salivary gland*	0	1	0	0	1	0.41
	Buccal mucosa	2	0	0	0	2	
	Tongue	3	1	0	0	4	
	Oropharynx	1	0	0	1	2	
	Jaw	1	0	1	0	2	
	Gingiva	0	1	0	0	1	

Table (4.3) Distribution of mutation of P53 and pRb by tumor behavior:

Markers	***Tumor***		***Total***
P53	***Malignant***	***Benign***	
positive	34(17%)	0(0.0%)	34(17%)
Negative	66(33%)	100(100%)	166(83%)
Total	100(50%)	100(50%)	200(100%)
Rb			
Positive	25(12.5%)	0(0.0%)	25(12.5%)
Negative	75(38%)	100(100%)	175(87.5%)
Total	100(50%)	100(50%)	200(100%)

P. value ≤ 0.001.

Table (4.4): Correlation of mutations in tumor suppressor genes (p53, Rb) and oral lesions with HPV infection.

Lesion behavior	P53 mutation	Rb mutation	Total
Malignant	3/10 (30%)	1/10 (1%)	4/10
Benign	0/2 (0.0%)	0/2 (0.0%)	0/2

P. value=0.497

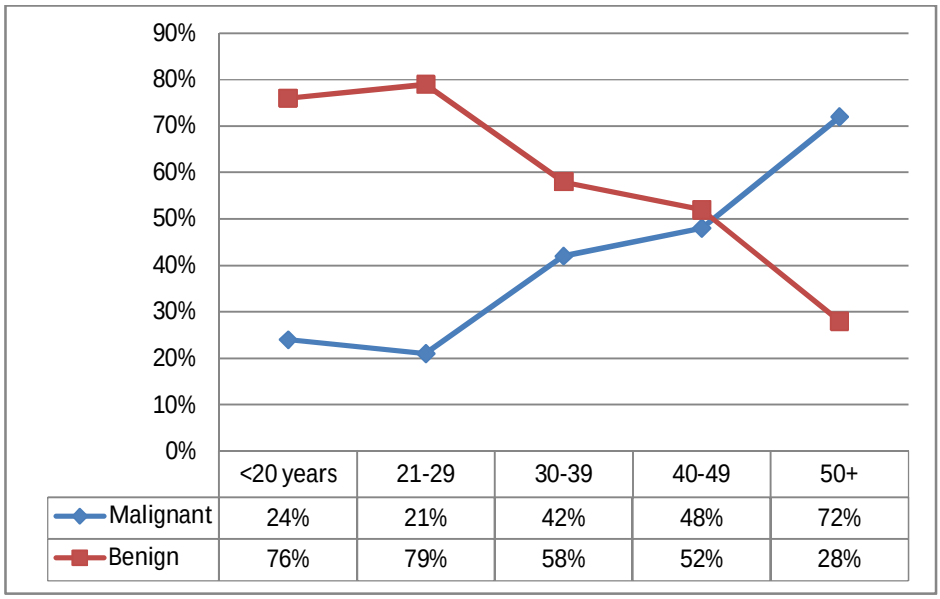


Figure (4.1). Description of the study population by age

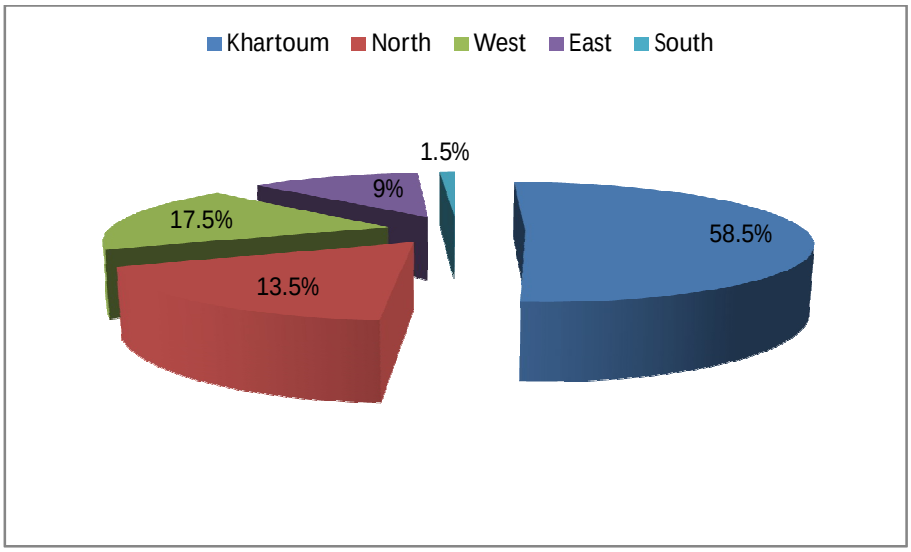


Figure (4.2) Description of of oral lesions according to residence of study population

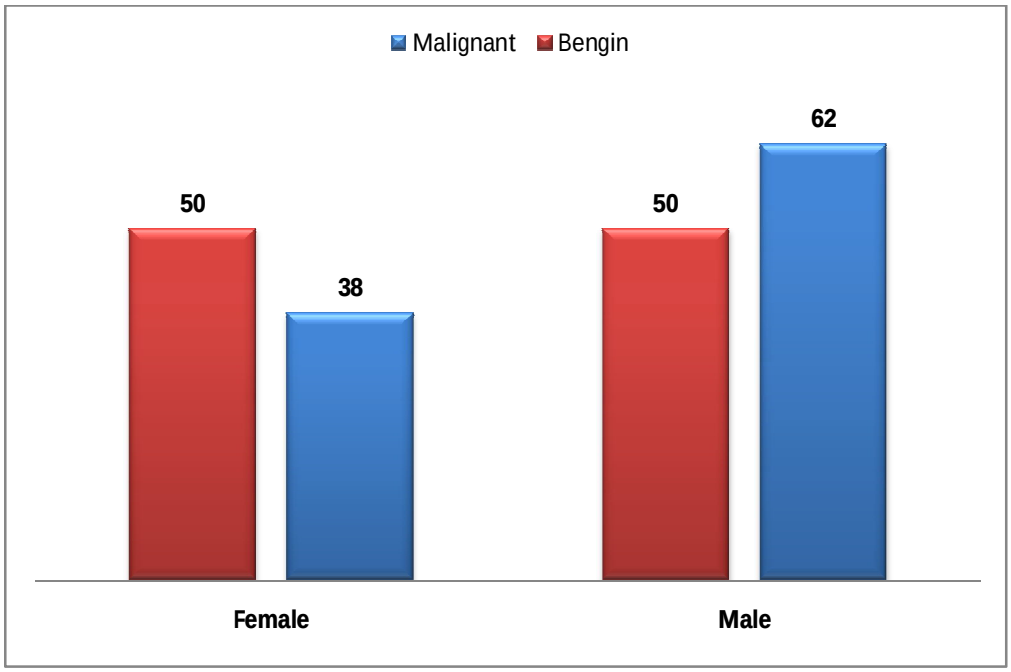


Figure (4.3): Description of the study population by oral lesions and gender.

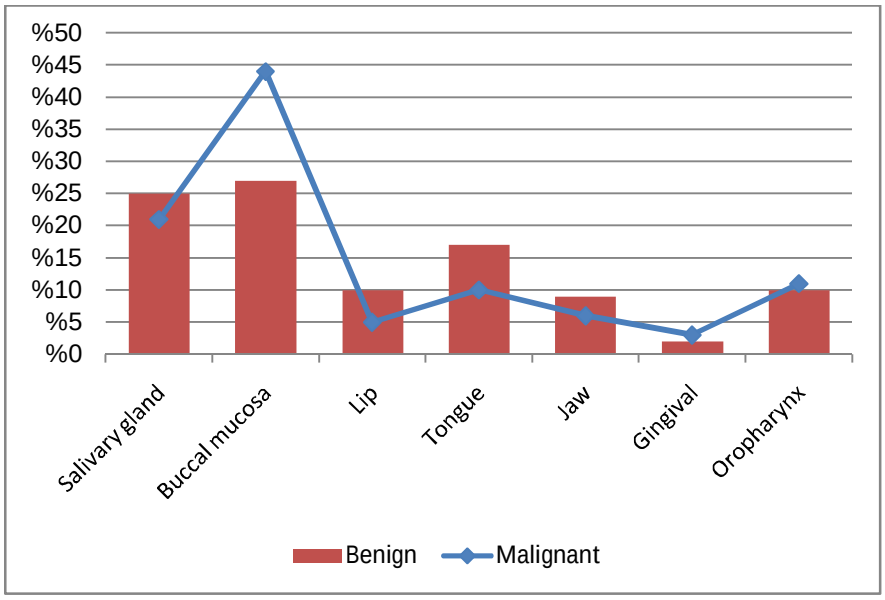
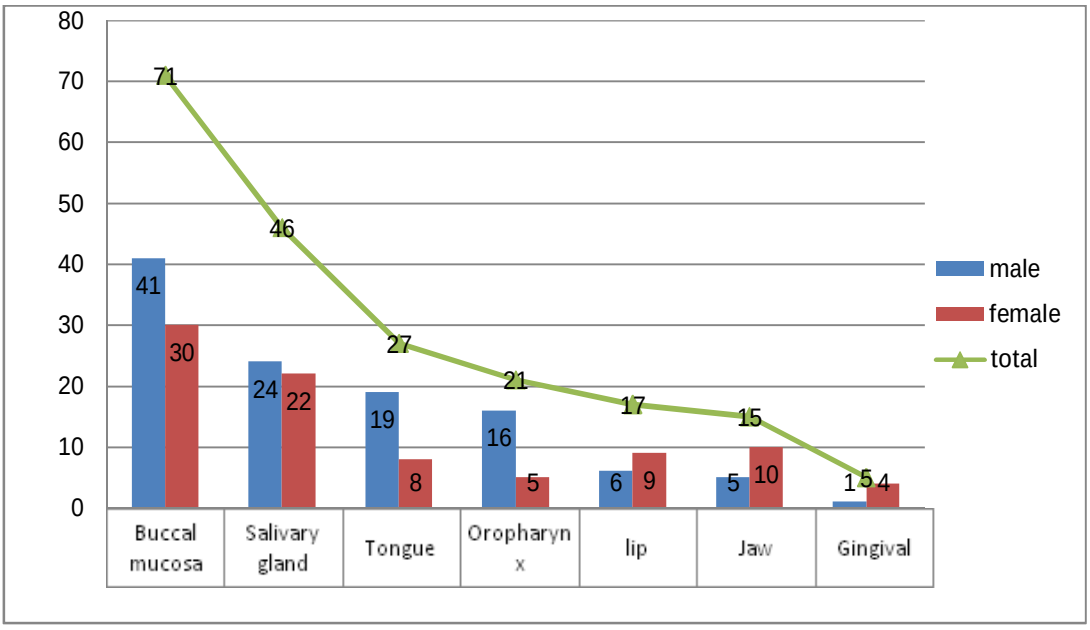


Figure (4.4): Description of study population by lesion sites and tumor type.



Figuer (4.5): Description of study population by lesion sites and gender.

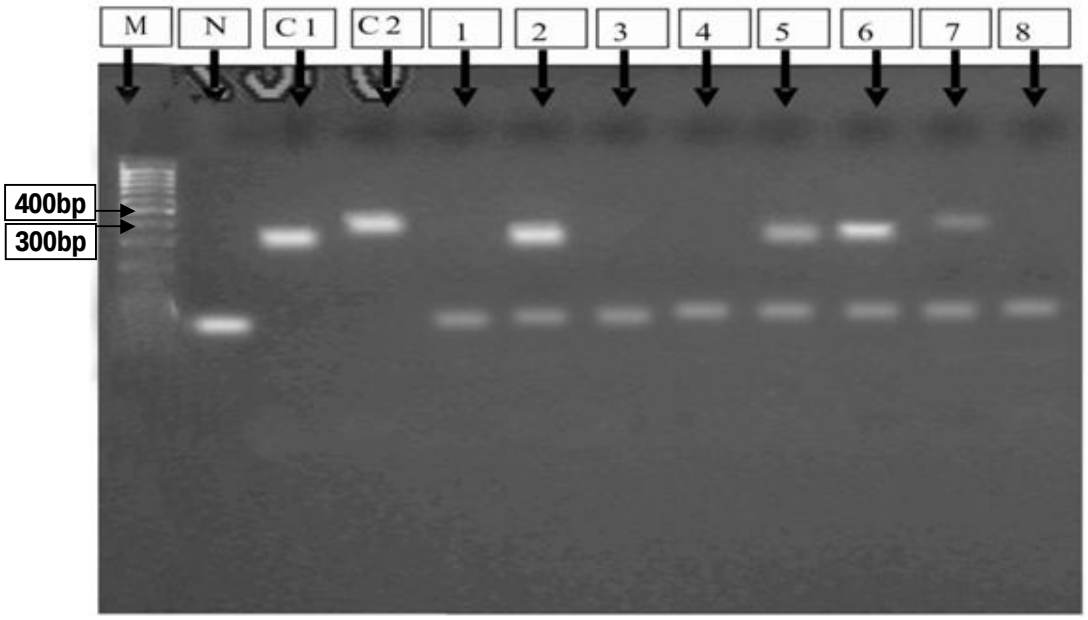
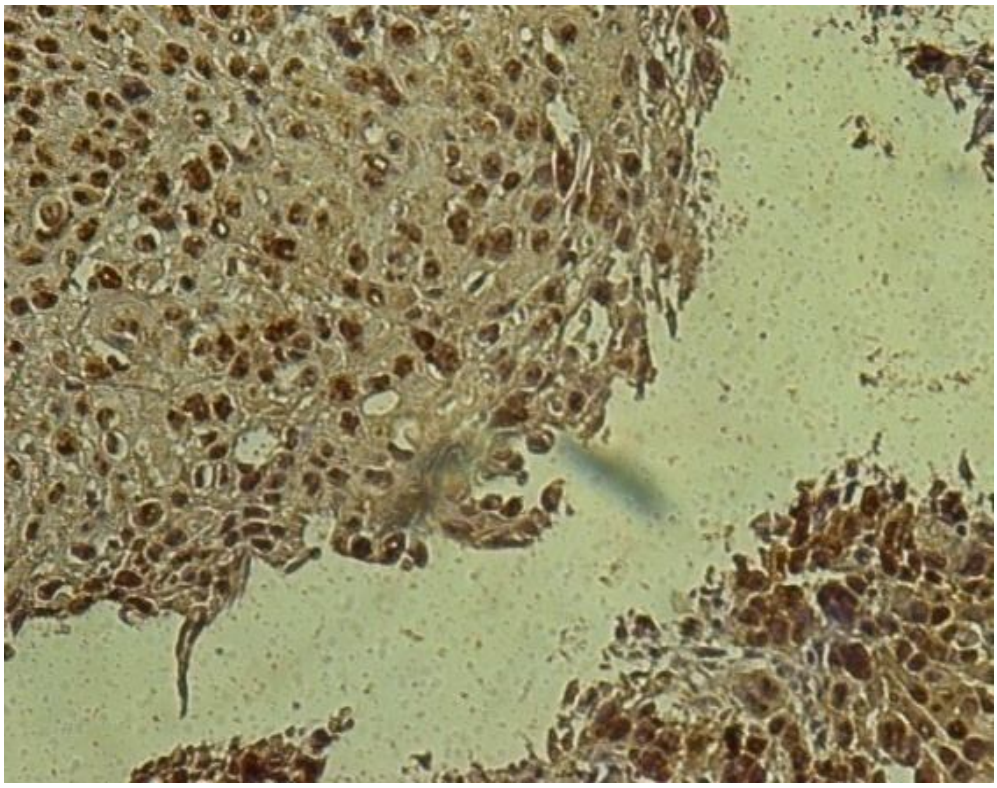
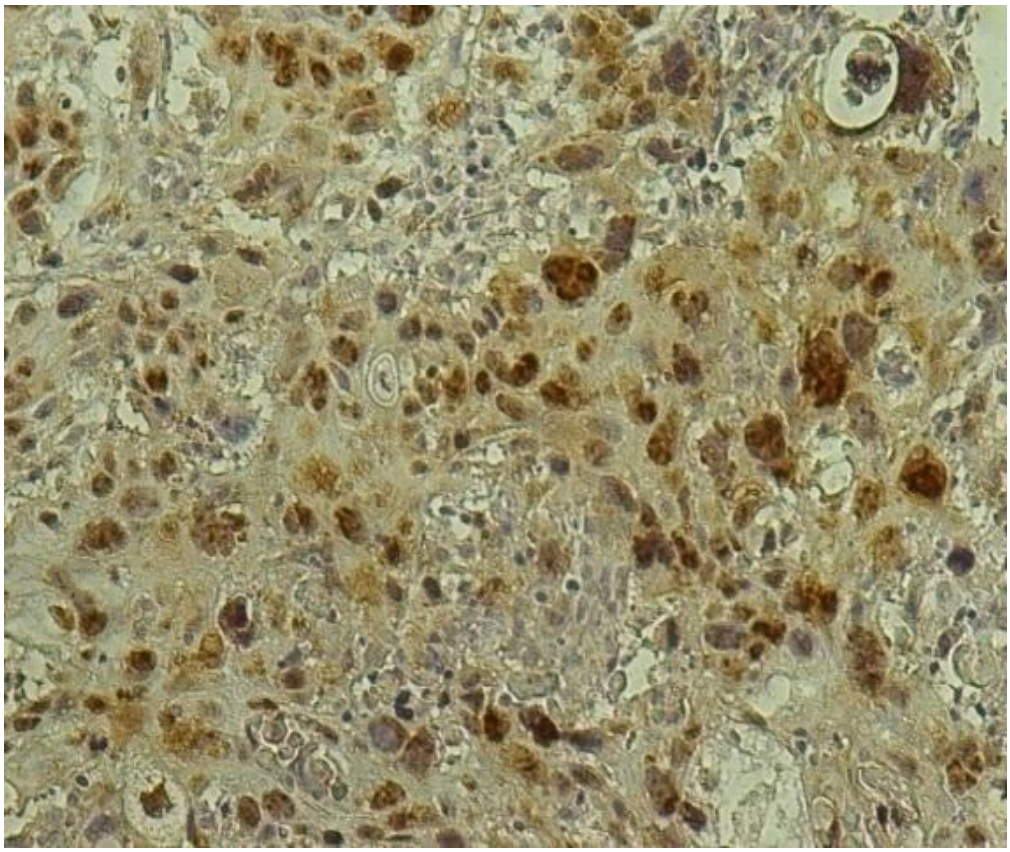


Figure (4.6). PCR amplification of HPV in oral lesions samples. The products were electophoresed on 2% agarose gel and stained with ethidium bromide.Lane M:1000bp ladder, (Arrows shows 300 and 400 band), Lane N negative control, Lane C1 positive control for HPV16, Lane C2 positive control for HPV18, lane 2,5,6,7. positive tumor samples, lane 2,5,6 HPV 16 positive tumor samples. Lane 3,4,8 negative samples. Lane 7 HPV 18. positive tumor samples.



Photomicrograph (4.1)

OSCC: P53 mutation Immunohistichemical staining 40 x: Strong positive.



Photomicrograph (4.2)

OSCC: Rb mutation Immunohistichemical staining 40x: positive

Chapter Five

Discussion

The epidemiological association of HPV with oral cancer is well established (Anaya, *et al.* 2008; Oliveira, *et al*. 2009). The prevalence of oral carcinomas reported to be associated with HPV has widely varied. Awide variations in spread the HPV between developed and developing countries in different population (Liang, *et al*. 2008). in this study, oral squamous cell carcinoma (OSCC) represented about 90% of oral cancer cases. Similar findings have been reported in several studies. The study by Jemal, *et al.* (2008) reported that OSCC represents more than 90% of all oral cancer cases. In another study by Palka, *et al.* (2008) they found that, the predominance of oral cancer are squamous cell carcinoma (SCC), which comprises greater than 90% of all oral cancer arising in those regions.

In the present study, HPV was detected in 10% of oral cancer and 2% of benign oral lesions. These results disclose a significant association between HPV and oral cancer, the P-value<0.05. However, similar studies from Africa showed higher, such as, the study by Laantri, *et al*. (2011) from Morocco, they assessed 62 cases of OSCC and identified HPV infection in 34% of the cases. Furthermore, relatively similar reports were found in studies from different countries, Chau, *et al.* (2011) assessed 53 cases of oral cancer for HPV infection and found 17/53 (32%) were positive. This little variation from our findings might be attributed to their small sample size compared to ours. The presence of HPV in oral cancer was also investigated by Weinberger, *et al*. (2010) they assessed 102 cases of oral cancer and identified HPV in 33/102 (32.4%) of the cases. Another study by Smith, *et al.* (2010), screened 210 oral cancer cases, HPV infection was revealed in 38% of the cases. However, in comparison with some regions over the world, the prevalence

of HPV in oral cancer in Sudan is low. In the developed countries the prevalence of HPV in oral cancer may exceed 30% particularly in young population, and this is attributed to oral sex. HPV is now the major cause of oropharyngeal cancer in developed countries, detected in 45–90% of cases (D'Souza *et al*. 2007; Kreimer, *et al*. 2005; Nasman, *et al.* 2009), but in Sudan no available studies showed the relation between oral sex and oral cancer, since, this is religiously and socially prohibited.

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 7 cases, 18 in 3 cases, and type 31 and 33 was found in one case for each. These findings are consistent with study by Syrjänen (2005) reported that, HPV16 is the most common genotype in oral cancers. Munoz, *et al*. (2003) reported that, HPV16, 18, 33, as high risk viruses, are frequently detectable in oral squamous cell carcinoma. HPV16, the most common HR-HPV type detected in biopsies from women with cervical SCC (55%), was also the most common type detected in biopsies from oral cancer (85-95%) (Munoz, *et al*. 2003). In the oropharynx, HPV16 accounted for the over whelming majority of HPV-positive cases (86.7%) (Munoz, *et al*. 2003). The other oncogenic HPV types 18 and 33 are also detected in invasive cervical cancer biopsies were also detected in oral cancer biopsies (Kreimer, 2005; Termine, 2008). Another study conducted in France by Guily, *et al.* (2011), they assessed HPV genotypes distribution in oropharyngeal and oral cancers. 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, which was similar to our findings. A study from Sudan evaluated the possible role of high risk HPV 16 and 18 in oral squamous cell carcinomas, 40 SCCs and 15

benign lesions, HPVDNA was detected in 15% of cases (six out of 40 cases), and none of controls (n=15), P<0.0001 (Ahmed and Altom, 2010). These findings were relatively close to the results reported by this study. A recent study from Sudan by Ginawi, *et al.* (2012) studied HPV types 16 and 18 among Sudanese patients with oral lesions, reported that, out of the 50 patients with OSCCs, 10(20%) were found positive for HPV types 16 and 18. 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 this result, which might be due to their small sample size.

In regard to a correlation between P53 mutations and oral cancer, we found (34%) mutations in oral cancer; statistically there is significant association between oral cancer and p53 gene mutation, the P.value:≤ 0.001. Similar results were published by Mao; *et al*. (2012) when they screened 50 cases of oral cancer for p53 mutation using immunohistochemical methods, (30%) of the cases showed positive P53 immunohistochemical staining. Another study from Italy conducted by Calzolari, *et al*. (1997) also screened P53 mutation in 85 cases of head and neck cancers, 35(41.2%) of them showed positive P53 mutation immunohistochemical staining. In India, Rowley, *et al*. (1998) assessed P53 in 19 cases of oral cancers (55%) of the cases disclosed positive P53 mutation immunohistochemical staining. Regarding the association between Rb gene mutation and oral cancer, Rb mutation was detected in 25% of oral cancer in this study, our findings support previous study in China, by Huang, *et al.* (2000), when screened 30 cases of oral cancer for mutation of Rb gene, (26%) of these cases found mutation of Rb gene. Another study by Sartor, *et al*. (1999) when screened 25 cases of OSCC for Rb mutation using immunohistochemical methods, (56%) of the cases showed positive Rb

mutation. The findings of our study were relatively lower than the results reported in previous studies, which might be due to their small sample size.

HPV infection has been demonstrated to play a role in the molecular pathways through its viral oncoproteins, E6 and E7. These proteins increase degradation of p53 and interfere with pRb function (Andl, *et al*. 1998).

In regard to the association between mutations of tumor suppressor genes (p53, Rb) and HPV in oral cancer, we found that 3/34(8.8%) of the p53 positive cases and 1/25(4%) of Rb positive were also HPV positive. Statistically, there is no significant association between tumor suppressor genes mutations (p53, Rb) and HPV in oral cancer. Similar results were published by Wrede, *et al*. (1991), that results are not significant of Rb and p53 genes mutations in HPV-positive and cervical carcinoma. Other results were published by Koh, *et al.* (1998) when screened 42 cases oral squamous cell carcinomas (SCCs) were analysed for p53 mutations and human papillomavirus (HPV) infection, (38%) of the cases showed positive P53 and negative with HPV. The state of the p53 gene mutation did not show any correlation with HPV. Another study from Indonesia conducted by Adi, *et al*. (2013) also screened eleven samples obtained from patients with OSCC who were positive HPV showed 18.2% had mutations in the p53 gene. Statistically insignificant found between the OSCC with HPV infection and the presence of p53 mutation.

In this study, most of the positive cases were identified in buccual mucosa, tongue, jaw, salivery gland, gingiva and oropharynx sites, and most common types were HPV16 and HPV18. These findings support other studies conducted in different parts of the world with HPV in many sites of oral region particularly tongue and oropharynx. However, HPV infection has been found to be strongly associated

with oropharyngeal cancer (Gillison, *et al.* 2000; Herrero, *et al*. 2003; Gillison, 2004).

In this study, Oral HPV is widely seen in the population of age group range from 30-50 years.These findings is similar to the studies by Shibosk, *et al.* (2005) and Hammarstedt, *et al*. (2006) when they reported that the incidence of oral cancers is progressively increasing, particularly among younger age groups, which may be at least partially attributed to HPV infection. Moreover, most oral squamous carcinoma (OSCC) occurs in older people, an increasing number of young patients are being affected worldwide, with up to 5.5% <40. These are predominantly oral and oropharyngeal cancers. Some patients have heavy exposure to the usual risk factors, but an increasing number do not. Part of this trend appears to be due to rising numbers of HPV associated oral cancer, particularly in males (Toner and O'Regan, 2009). Though, there are epidemiological differences between HPV-DNA-positive and HPV-DNA-negative oral cancer, HPV-DNA-positive cancers are associated with younger age and higher numbers of sexual partners, but are less associated with tobacco smoking compared with HPV-DNA-negative cancers (Chaturvedi *et al*. 2008; Gillison *et al*. 2008; Heck *et al*. 2010).

Most patients with positive HPV were from Khartoum. This might be attributed to the largest population on Khartoum, with varying socioeconomic status and behavioural differences.

There are clear limitations it is retrospective setting that lack identification of some demographical and exposure data such as patient's socioeconomic status, nutritional status, previous health neither history nor family relations. A major limitation of our study is the lack of information regarding alcohol intake and smoking habits.

5.1 Conclusion and Recommendations

5.1.1 Conclusion

The findings of the present study suggest that:

1. The most prevalent oral cancers in Sudanese patients were squamous cell carcinoma which represents 90% of all oral lesions.
2. There is high frequency of tumor suppressor genes mutations (P53, Rb) among Sudanese patients with oral cancer.
3. There is no statistically association between HRHPV and tumor suppressor genes mutations (P53, Rb) in this study.

5.1.2. Recommendations

1. Further studies with large sample size are recommended to measure the real burden of HPV in etiology of oral cancer in Sudan.
2. The exact causes of mutations in tumor suppressor genes require further investigation.
3. Screening for the relationship between oral cancer and low risk HPV is required.

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Chapter Six

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APPENDIX

ITEM	Quantity
Haematoxilin and eosin kit	2 kits
Real Envision Detection Kit	2 kits
primary mouse monoclonal mutant Rb antibody	0.4 ml
primary mouse monoclonal mutent p53 antibody. From (Gene tech company limited, Shanghai, China)	0.4 ml
DNA extraction from *Sacace technologies- Casera –Italy*)	4 kits
Hgh risk HPV kit (from *Sacace technologies- Casera –Italy*)	4kits
Xyline	I liter
Ethanol	2liter