Chapter One

Introduction

1.1 Cervical Cancer

Cervical cancer is the third most common cancer among women worldwide (Ferlay *et al.,* 2010 and GLOBOCAN, 2008-http://onlinelibrary.wiley.com/doi/10.3322/caac.20107/full?dmmsmid=71827&dmmspid=19396336&dmmsuid=1908926). Cervical cancer is an abnormal growth of the cells of the cervix. The cervix is the lower part of the uterus that opens into the vagina. The cervix covered (lined) by squamous cells on the exocervix (towards vagina) and glandular cells on the endocervix (towards uterus) (Plate 1.1A). These two types of cells meat at the transformation zone where most cervical cancers start (Plate 1.1B) (http://www.cancer.org/cancer/cervicalcancer/detailedguide/cervical-cancer-what-is-cervical-cancer). Mucosal human papillomavirus (HPV) is a necessary cause of cervical cancer (Bernard, *et al.,* 2013).

1.2 Symptoms

Early cervical cancer or abnormal cervical cell changes (Precancerous lesions) are generally asymptomatic. As the cancer progresses, the following symptoms may appear as; vaginal discomfort,

vaginal and pelvic pain or bleeding after sex, between periods or after menopause, watery bloody malodorous vaginal discharge that may be heavy and have a foul odor (http://www.mayoclinic.org/healthy-living/sexual-health/in-depth/cervical-cancer-vaccine/art-20047292).
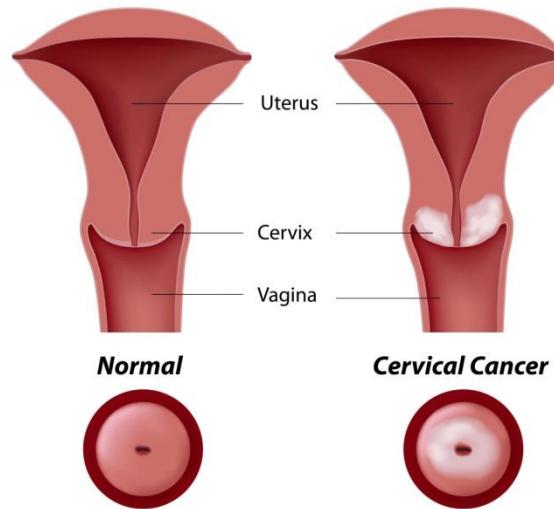
Plate 1.1A The normal cervix and cervical cancer(vertical)

http://intimatehealthhelp.net/wp-content/uploads/2012/08/cervical-ancer.jpg.
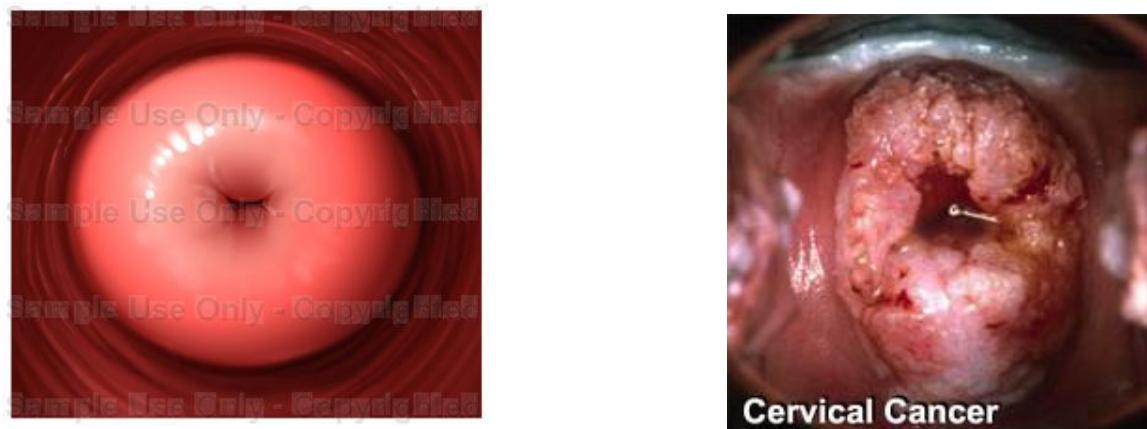
Plate 1.1B   The normal cervix and cervical cancer

http://graphicwitness.medicalillustration.com/imagescooked/36633W.jpg.

Plate1.2 Cervical squamous cell carcinoma

http://library.med.utah.edu/WebPath/FEMHTML/FEM013.html.

The symptoms of advanced cervical cancer may include: loss of appetite, weight loss, fatigue, pelvic pain, back pain, leg pain, swollen legs, heavy bleeding from the vagina, bone fractures, and/or (rarely) leakage of urine or feces from the vagina (Plate 1.1B and 1.2) (Sichanh *et al,* 2014).

1.3 Epidemiology

Cervical cancer is the third most common cancer among women worldwide. About 530,000 women contracted cervical cancer annually, leading to death of 288,000 women every year (Ferlay *et al.,* 2010; WHO, 2010 and Baseman *et al*., 2005). Cervical cancer spread more in the third world and decreases in USA and Europe. The United States Centres for Disease Control and Prevention (CDC) has classified cervical cancer, Kaposi sarcoma and non-Hodgkin's lymphoma as AIDS-defining cancers. (Mbulaiteye *et al.,* 2011 and Ononogbu *et al.,* 2013)

In Sudan the cervical cancer is the third most common cancer type (n, 212) among women in Sudan in 2009 - 2010 according to the Sudanese National Population-based Cancer Registry (NCR) (Saeed *et al.,* 2014).

1.4 Incidence

The High-risk regions for Cervical Cancer are East and West Africa with age-standardized incidence rate (ASR) greater than 30 per 100,000, followed by Southern Africa (26.8 per 100,000), South-Central Asia (24.6 per 100,000), South America and Middle Africa (ASRs 23.9 and 23.0 per 100,000 (Ferlay *et al*., 2010). Lower rates are in Western Asia, Northern

America and Australia/New Zealand (ASRs less than 6 per 100, 000) (Ferlay *et al.*, 2010) Some Eastern and Central European countries have relatively high incidence such as Serbia (Tavassoli *et al.*, 2003), where the ASR of Cervical cancer is 27.2 per 100,000 - twice as high as in Western European countries (Matejić *et al.*, 2008).

The highest age-standardized incidence rates of Cervical cancer have been reported in Malaysia, Southern Africa, Central America, Eastern Africa, and South America, where the rates were over 40 per 100,000 at each religion and the age-standardized mortality rate from Cervical cancer in these countries is 9.6 per 100,000 women, twice the rate in developed countries (WHO, 2010).

In sub Saharan African countries, the age-standardized incidence of Cervical cancer is two to ten times higher than that in devolved countries 30 to 67 per 100,000 (Debbie *et al.*, 2007). The incidence of cervical cancer varies widely among countries with world age-standardized rates ranging from <1 to >50 per 100 000 (Arbyn *et al,* 2011).According to the Sudanese National Cancer Registry 2009 -2010, the incidence of cervical cancer in Sudan was found to be 7.4 per 100,000 in Sudanese women (Saeed *et al.,* 2014).

1.5 Mortality of Cervical cancer

Cervical cancer is the second most common cause of death from Cancer among young women, accounting for nearly 300,000 deaths annually Breast cancer as the most common cause of death from cancer in women worldwide and in Sudan (Jin *et al.*, 1999 and Saeed, 2014).

In Africa about 53,000 women die of the cervical cancer annually and the mortality rates is 34.6   at East Africa (WHO, 2010). In USA an

estimated 12,800 women are still diagnosed each year with invasive Cervical cancer and approximately 4,600 will die of their disease (Greenlee *et al*., 2000). In Sudan the Age-standardized mortality rate is 4.9 and the crude mortality rate is 3.0 (IARC, Globocan 2008)**.**

1.6 Types of cervical cancer

Almost all cervical cancers are either squamous cell carcinomas or adenocarcinomas, however few other types of cancer also can develop in the cervix.   Most cervical cancers begin in the cells lining the cervix. These cells do not suddenly change into cancer. Instead, the normal cells of the cervix first gradually develop pre-cancerous changes that turn into cancer. Pre-cancerous changes are abnormal cellular changes and growth on the surface of the cervix, that may become cancerous, and these could be detected by Pap test (www.cancer.org/cancer/cervicalcancer/detailedguide/ cervical-cancer-what-is-cervical-cancer). These changes termed as; cervical intraepithelial neoplasia (CINI, II, III & IV), squamous intraepithelial lesion (SIL) (Debbie *et al*., 2007 and Baak *et al*., 2006).

1.6.1 Cervical Squamous Cell Carcinoma

Approximately 90% of cervical cancers are squamous cell carcinomas in the world and Sudan (Husain *et al.,* 2011). This type of cancer originates or develop in the thin, flat, squamous cells on the surface of the ectocervix, the part of the cervix that is next to the vagina (Debbie *et al*.,2007 and Baak *et al*., 2006). It develops gradually through well-characterized precursor lesions squamous intraepithelial lesions-SILs. Most cervical cancers arise at the squamo-columnar junction and transformation zone between the columnar epithelium of the endocervix

and squamous epithelium of the ectocervix where continuous metaplastic changes occur (Plate 1.3) (Debbie *et al*., 2007 and Baak *et al*., 2006).

1.6.2 Cervical Adenocarcinoma

Adeocarcinoma (10%) is the second most common form of cervical cancer. Cervical adenocarcinoma develops from the mucus-producing gland cells of the endocervix, near the body of the uterus (http://medical-dictionary.thefreedictionary.com/Carcinoma+of+the+cervix). Their precursor lesions, are located high in the endocervical parts of the transitional zone. Global estimates indicate that adenocarcinomas now comprise up to one quarter of cervical cancer cases in some Western countries (Freddie *et al*., 2005 , Xavier *et al*., 2006).

1.7 WHO and Bethesda classification system

The initial changes that may occur in some cervical cells are not cancerous. However, these precancerous cells form a lesion called dysplasia or a squamous intraepithelial lesion (SIL), since it occurs within the epithelial or outer layer of cells. These abnormal cells can also be described as cervical intraepithelial neoplasia (CIN). Moderate to severe dysplasia may be called carcinoma in situ or non-invasive cervical cancer (Fig.1.1 and 1.2).

Dysplasia is a common condition and the abnormal cells often disappear without treatment. However, these precancerous cells can become cancerous. This may take years, although it can happen in less than a year. Eventually, the abnormal cells start to grow uncontrollably into the deeper

layers of the cervix, becoming an invasive cervical cancer (http://medical-dictionary.thefreedictionary.com/Carcinoma+of+the+cervix.2/11/2014).

Cervical intraepithelial neoplasia - CIN is the potential precursor to cervical cancer, is often diagnosed on examination of cervical biopsies by a pathologist. For premalignant dysplastic changes, the CIN grading is used. WHO classification system was descriptive of the lesions, naming them mild, moderate or severe dysplasia or carcinoma in situ (CIS). The term, Cervical Intraepithelial Neoplasia (CIN) was developed to place emphasis on the spectrum of abnormality in these lesions, and to help standardize treatment. It classifies mild dysplasia as CIN1, moderate dysplasia as CIN2, and severe dysplasia and CIS as CIN3. More recently, CIN2 and CIN3 have been combined into CIN2/3. These results are what a pathologist might report from a biopsy (Fig.1.1 and 1.2).

WHO system should not be confused with the Bethesda System terms for Pap smear (cytopathology) results. Among the Bethesda results: Squamous intraepithelial lesions (SILs) divided into Low-grade Squamous Intraepithelial Lesion -LSIL (mild abnormal cells) and High-grade Squamous Intraepithelial Lesion -HSIL (severely abnormal cells that develop to cancer). An LSIL Pap may correspond to CIN1, and HSIL may correspond to CIN2 and CIN3 (Fig.1.1. and 1.2).

1.8 Stages of cervical cancer

Stage I: Cancer is confined to the cervix and no spread to nearby tissues.

Stage II: Cancer includes the cervix and vagina but hasn't spread to the pelvic side wall or the lower portion of the vagina.

Stage III: Cancer has moved and spread beyond the cervix to the pelvic side wall or the lower portion of the vagina.

Stage IV: In this stage cancer has spread to nearby organs (metastasis), such as the bladder or rectum, or it has spread to other areas of the body, such as the lungs, liver or bones.

1.9 Cytological diagnosis; Papanicolaou test - Pap test

The Pap test (or Pap smear) is a procedure used to collect cells from the cervix for cervical cytology testing. Cervical cancer is first detected with a Pap test that is performed as part of a regular pelvic examination. The Pap test is a screening tool rather than a diagnostic tool. It is very efficient at detecting cervical abnormalities (Plate 1.4). .

The vagina is spread with a metal or plastic instrument called a speculum to keep it open so that the cervix can be seen clearly. A swab is used to remove mucus and cells from the cervix. A small brush or a cotton-tipped swab is then inserted into the cervical opening to take a sample from the cervix. The cell samples are then prepared for microscopic examination (http://www.cancer.org/cancer/cervicalcancer/detailedguide/ cervical-cancer-what-is-cervical-cancer) and is sent for microscopic examination. A Pap test can detect abnormal cells in the cervix, including cancer cells and cells that show changes (dysplasia) that increase the risk of cervical cancer (Plate 1.4) .

According to The Bethesda System (TBS), the Pap results are reported into three general categories; negative for intraepithelial lesion or malignancy, or epithelial cell abnormalities (cancer or a pre-cancerous condition) or other malignant neoplasms.
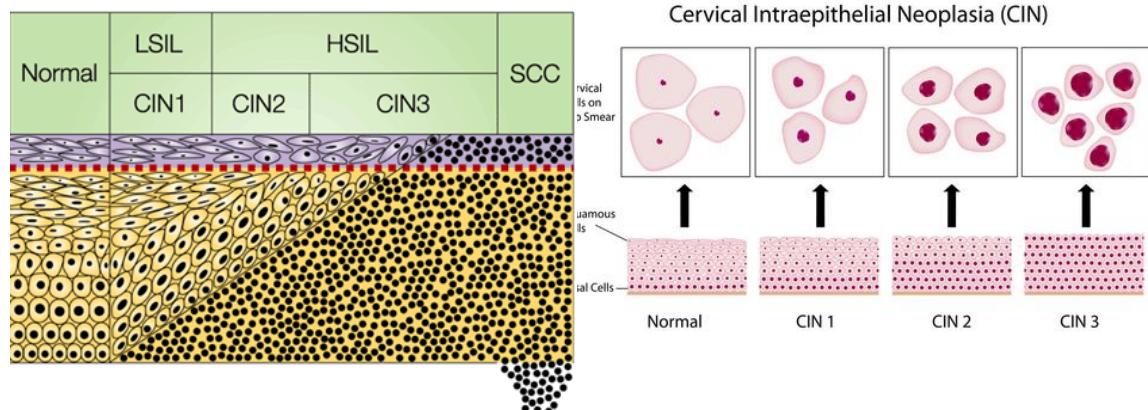
Figure 1.1 Cervix normal cells and cervical intraepithelial neoplasia (CIN) .
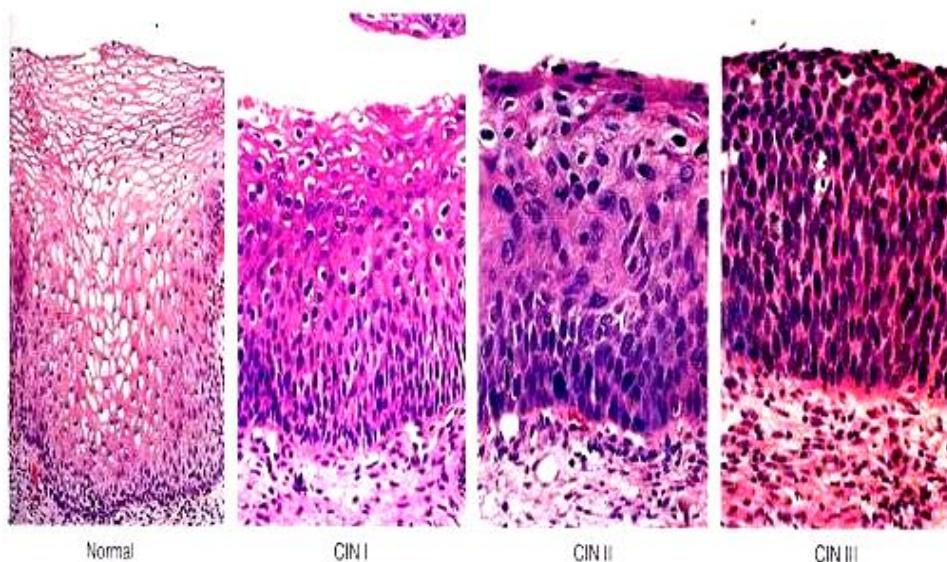
**Figure 1.2 Histopathology of the normal cervix and CIN.**
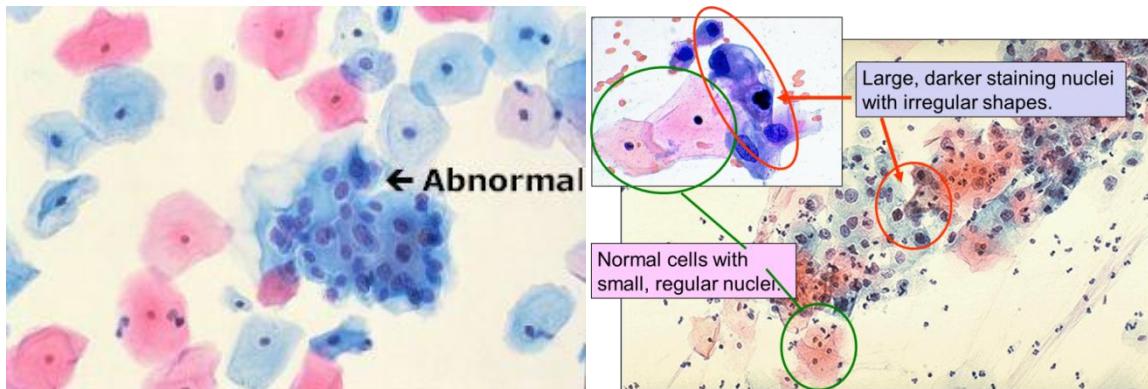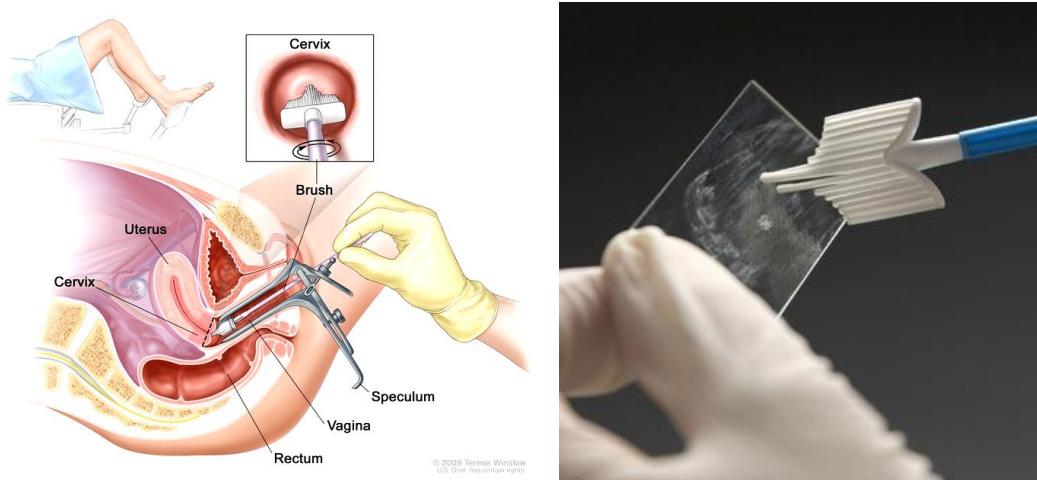
Plate 1.3   Pap test: Sample collection, smear preparation   and microscopic examination

1.10 Treatment

Treatment for cervical cancer depends on ; the stage of the cancer and patient's other health problems preferences about treatment. Noninvasive intraepithelial lesions are treated with cryotherapy, cold knife conization, laser therapy or loop electrosurgical excision procedures (Burd, 2003). Treatment options may include:

- **Surgery.**
- Surgery to remove the uterus (hysterectomy) is typically used to treat and to cure the early stages of cervical cancer and prevent cancer from coming back.

  A simple hysterectomy involves the removal of the cancer, the cervix and the uterus, when the cancer is at a very early stage and an invasion is less than 3 millimeters (mm) into the cervix.

  A radical hysterectomy — removal of the cervix, uterus, part of the vagina and lymph nodes in the area, when there's invasion greater than 3 mm into the cervix.

- **Radiation.**
- Radiation therapy uses high-powered energy to kill cancer cells. Radiation therapy can be given externally using external beam radiation or internally (brachytherapy) by placing devices filled with radioactive material near the cervix, or combined. Radiation therapy can be used alone, with chemotherapy, before surgery to shrink a tumor or after surgery to kill any remaining cancer cells.

- **Chemotherapy.**

  Chemotherapy uses drugs to kill cancer cells. Chemotherapy drugs are usually injected into a vein, and they travel throughout the body killing rapidly growing cells, including cancer cells. Low doses of chemotherapy are often combined with radiation therapy, since chemotherapy may enhance the effects of the radiation. Higher doses of chemotherapy are used to control advanced cervical cancer that may not be curable (ER.1).

1.11 Vaccination

Widespread HPV immunization, however, could reduce the impact of cervical cancer worldwide. There are two cervical cancer vaccines, Gardasil and Cervarix. Quadrivalent Merck Gardasil® vaccine ((Merck, Whitehouse Station, NJ) protected against cervical cancer as well as genital warts, and offer protection against HPV16, 18, 6 and 11 (Gardasil, 2012; Medeiros *et al* ., 2009). Gardasil was licensed in the UK in September 2006 for girls and women aged between 9 and 26. Girls have three injections over 6 months http://www.mayoclinic.org/healthy-living/sexual-health/in-depth/cervical-cancer-vaccine/art-20047292 It's cost was £400 in private clinics. vaccination gives protection for at least 20 years. The vaccine is protective only after completing the whole course. But the vaccine won't get rid of already exists HPV infection. At this time it is unknown that, if Gardasil vaccine could prevent other types of cancer (Gardasil, 2012).

Cervarix® (GlaxoSmithKline, Research Triangle Park, NC), this bivalent vaccine protects against HPV types 16 and 18 and was licensed in the UK in 2007 and in 2009 in USA for the prevention of pre cancerous

changes in the cervix in girls and women between the age of 10 and 25 (FDA, 2012).   .

1.12 Risk factors

Several risk factors increase the chance of developing cervical cancer, however some may not develop cervical cancer.

1.12.1 Human papilloma virus infection

The most important risk factor for cervical cancer is infection by the human papilloma virus (HPV), which may develop non malignant growth called a *papilloma (warts)* on the skin (cutaneous) or mucosal (genital) parts of the body. However, approximately 90% of HPV infections clear on their own within months to a few years. Cutaneous HPVs infect cells on the surface of the skin causing cutaneous warts. Mucosal or genital HPVs infects the cells lining the genitals, anus, mouth and throat and causing *genital warts* or *condyloma acuminatum*. HPV is spread is by sex and skin contact (zur Hauzen, 2000).

Women harboring HPV 16 with normal cervical cytology have over a 100 times increased risk of developing CIN III compared with HPV negative women, and progression to CIN III and invasive disease is strongly associated with persistence of "high risk" viral types (HPV16 and HPV18). About 70% of cervical cancers caused by HPV16 and 18 (zur Hausen, 1996).

High-risk HPV types because they are strongly linked to cancers, including cancer of the cervix, vulva, and vagina in women, penile cancer in men, and cancers of the anus, mouth, and throat in both men and women.

The high-risk types include HPV; 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 (Bouvard *et al.,* 2009). About two-thirds of all cervical cancers are caused by HPV 16 and 18. There might be no visible signs of infection with a high-risk HPV until pre-cancerous changes or cancer develops. Cervical cancer caused by chronic infection by high risk HPVs. The Pap test looks for changes in cervical cells caused by HPV infection. HPV detected by DNA examination by molecular techniques. Although there is currently no cure for HPV infection.

1.12.2 Smoking

smoking is the most important independent risk factor of HPV infection for higher grades of Cervical disease (Adam *et al.*, 2000). The association between tobacco smoking and Squamous Cell Carcinoma has been demonstrated (Sturgis *et al.,* 2004). Smoking may be the most important independent risk factor of HPV infection for higher grades of cervical disease (Adam *et al.*, 2000).

Women who smoke were associated with an increased risk for developing cervical squamous cell carcinoma, about four times as likely as non-smokers. Tobacco by-products have been found in the cervical mucus of women who smoke, which may damage the DNA of cervix cells and may contribute to the development of cervical cancer (Sturgis *et al.,* 2004). Smoking also makes the immune system less effective in fighting HPV infections(http://www.cancer.org/cancer/cervicalcancer/detailedguide/ cervical-cancer-what-is-cervical-cancer.)

Many cancer-causing chemicals present in tobacco, and these harmful substances are absorbed through the lungs and carried in the bloodstream throughout the body (http://www.cancer.org/cancer/cervicalcancer

/detailedguide/ cervical-cancer-what-is-cervical-cancer.) Cigarette smoke contains over 4,000 chemicals, including 43 known cancer-causing (carcinogenic) compounds and 400 other toxins. These include nicotine, tar, and carbon monoxide, as well as formaldehyde, ammonia, hydrogen cyanide, arsenic, and DDT (http://www.quitsmokingsupport.com/whatsinit.htm).

1.12.3 Alcohol

The IARC of the WHO has classified alcohol as a Group 1 carcinogen (IARC, 1988). The exact way alcohol affects cancer risk isn't completely understood. In fact, there might be several different ways it can raise risk, and this might depend on the type of cancer.

Alcohol damaged the cells such as liver cells that may try to repair themselves, which could lead to DNA changes in the cells that could lead to cancer. The bacteria of the digestive system convert alcohol into the carcinogenic   acetaldehyde. Alcohol help harmful chemicals to enter the cells lining the digestive tract, also alcohol slow the body's ability to break down and get rid of harmful chemicals. Alcohol lower the body's ability to absorb foliate (vitamin) from foods, low foliate levels may lead to cancers. Alcohol raise body levels of hormones as estrogen, which is important in the growth and development of breast tissue. Too much alcohol can add extra   calories   to   the   diet,   and   increase   weight   (obese) (http://www.mayoclinic.org/healthy-living/sexual-health/in-depth/cervical-cancer-vaccine/art-20047292).

1.12.4 Sexual activity

HPV is a sexually transmitted virus. It poses a great risk when; partners began intercourse at young age ($\leq$ 16 years), sexual partner with multiple partners, and having sex with previous partner with cervical cancer (http://medical-dictionary.thefreedictionary.com/Carcinoma+of+the+cervix).

1.12.5 Oral contraceptives

Along-term use of oral contraceptives (steroids) is a significant risk factor for high-grade cervical cancer (Adam *et al.,* 2000; Brisson *et al.,* 1994 and Parikh *et al.*, 2003). Intrauterine devices are also a risk factor for developing cervical cancer (Zhang *et al.*, 1997).

steroid oral contraceptive use for more than five years has been reported to increase the risk of invasive cervical cancer twice among HPV infected women. HPV16 upstream regulatory region (URR) of the genome, mediates transcriptional control of the HPV genome and is thought to contain enhancer elements that are activated by steroid hormones It has a direct effect on HPV transcription and an indirect effect leading to increased levels of HPV oncogene expression (Sonnex, 1998).

Steroid contraception has been postulated to be one mechanism whereby HPV exerts its tumorigenic effect on cervical tissue. Steroids are thought to bind to specific DNA sequences within transcriptional regulatory regions on the HPV DNA to either increase or suppress transcription of various genes (Moodley *et al.*, 2003).

1.12.6 Genetic susceptibility

Inherited genetic predisposition may contribute to the risk of cervical cancer. Cervical cancer may run in some families. Women who have an affected first-degree biologic relative have a 2-fold relative risk of developing a cervical tumor compared with women who have a non biologic first-degree relative with a cervical tumor. Genetic susceptibility accounts for fewer than 1% of cervical cancers (Magnusson *et al.*, 1999 and Galloway, 2003).

Some researchers suspect that some instances of this familial tendency are caused by an inherited condition that makes some women less able to fight off persistent HPV infection than others. Genetic polymorphisms in tumor suppressor genes might be related to HPV persistence and progression to cancer (Storey *et al*, 1998). In other instances, women from the same family as a patient already diagnosed could be more likely to have one or more of the other non-genetic risk factors (Magnusson *et al.,* 1999 and Galloway, 2003).

1.12.7 Nutrition

Diets that are low in fruits and vegetables increase the risk of cervical cancer. Vitamin A is associated with a lower risk as is vitamin B12, vitamin C, vitamin E, and beta-carotene. Obese and overweight women were linked to increased risk for cervical adenocarcinomas. The link appears to be increase levels of estrogen. Excessive fat tissue influences levels of estrogen and other sex hormones (http://medical-dictionary.thefreedictionary.com/Carcinoma+of+the+cervix).

1.12.8 Parity

A direct association was found between the number of full-term pregnancies and squamous-cell cancer risk: the odds ratio for seven full-term pregnancies or more was 3·8 (95% Cl 2·7—5·5) compared with nulliparous women, and 2·3 (1·6—3·2) compared with women who had one or two full-term pregnancies. High parity increases the risk of squamous-cell carcinoma of the cervix among HPV-positive women (Auvert *et al*, 2009). Hormonal changes during pregnancy as possibly making women more susceptible to HPV infection or cancer growth, also pregnant women might have weaker immune systems, allowing for HPV infection and cancer growth. Pregnancy at young age also present a risk. A general decline in

parity might therefore partly explain the reduction in cervical cancer recently seen in most countries (Auvert *et al*, 2009 and Schiff *et al*., 2000).

1.12.9 Immunosuppression

Immunosuppressed (AIDS), immunocompromised patients and those with autoimmune disease and have cervical pre-cancer might deveop into an invasive cancer faster than it normally would. Immunosuppression is associated with the development of anogenital intraepithelial neoplasia appropriate compromised immune response to infection with human papillomavirus (http://medical-dictionary.thefreedictionary.com/Carcinoma+of+the+cervix).

1.12.10 Socio-economic factors

Worldwide poor women of low socio-economic status have a greater risk of having cervical cancer (Palacio-Mejia *et al.,* 2003). Many factor increase the problem in Sub Sahara Africa as; wars, political chaos, internal conflicts, natural disasters, famine and drought and low education Poverty is associated with poor health care system (Suba *et al.,* 2006).

Rationale

Cervical cancer is the third cause of death for cancer in women, and about 275000 new cases are reported annually. Cancer is a fatal disease with highly expensive treatment regiments from surgical to chemical and radiation. The number of cancer patients in Sudan is in increase. Few contradictory researches were published from Sudan. Sudan has limited capacity for cancer; diagnosis, treatment and expensive molecular cancer researches. Moreover, researchers are unwelcomed gusts by clinicians and pathologists.

This study aimed to detect the high risk Human papilloma viruses; HPV16 and HPV18, in Sudanese women with cervical squamous cell carcinomas. Also will link HPV16 and 18 with degree of tumor differentiation, age, sites and jobs.

Objectives

General objectives:

1- To detect *Human papillomavirus*   in Sudanese women with cervical cancer.

 Specific objectives:

1- To identify high risk HPV16 and HPV18 in cervical squamous cell carcinomas.

3- To link HPV, HPV16 and HPV18 with tumor differentiation, age, sites and jobs.

Literature Review

2 Human papillomavirus

2.1 HPV prevalence

About 15% of cancers can be linked to tumor viruses such as hepatitis HBV, HCV, Epstein-Barr virus, Human Papillomaviruses, human herpes virus type 8, human T-lymphotropic retrovirus type 1, Human immunodeficiency viruses type HIV-I and HIV-II. These agents contribute indirectly to human carcinogenesis (zur Hausen, 2001). Human papillomavirus is the major cause of cervical cancer (zur Hausen, 1996; 1999 and Gillison, 2003). HPV was detected in cancers of oral cavity, larynx, and esophagus (zur Hausen, 1996). Presence of HPV DNA within a human tumor represent a hint but clearly not proof for an etiological relationship (zur Hausen, 1999).

Human papillomavirus (HPV) is one of the most common sexually transmitted disease in the world (Cates *et al*., 1999). Most people in the world are probably infected with at least one if not several types of HPV during their life. The prevalence of HPV infection in world was estimated to be between 9 to 13%, and about 1.6 to 25.6% by country (Clifford *et al.,* 2005). In USA about 6.2 million people are infected every year with HPV, and at least 50% of all sexually active people might get infected with HPV throughout their lives (CDC, 2007).

2.2 Taxonomy of papillomavirus

Previous taxonomy of papillomaviruses was based on biological properties (de Villiers *et. al.,* 2004). The first completely sequenced genomes for classification were those of HPV 1 (Chen *et. al.,* 1982), HPV 11 (Danos *et. al.,* 1982), HPV 6 (Schwarz *et. al.,* 1983), and HPV 16 (Seedorf *et. al.,* 1985).

Classification of PV types based predominantly on L1 nucleotide sequence identities similarities with some biological and medical properties and their topological position within PV phylogenetic trees (de Villiers *et al.,* 2004; Fauquet *et al.*, 2005 and Bernard *et al.*, 2010). L1 encodes the late principle capsid protein (55000 Daltons).

L1 is highly conserved among different papillomavirus types and used for diagnosis, identification and vaccination. The L1 gene can be aligned for all known PVs. L1 is useful for classification construction of phylogenetic trees and PVs nomenclature (de Villiers *et al.,* 2004; Fauquet *et al.*, 2005).

The PV taxonomic levels are; "Family", "Genus"`, "Species", "types", "sub-types" and "variants". The international committee of the virus toxonomy (ICTV) define a species that it should be "a polythetic class of viruses that constitute a replicating lineage and occupies a particular ecological niche". A phylogenetic species concept would lead to a promotion of PV types (strains) to species, and of the present species to sub-genera. (Bernard *et al.,* 2010). PV subtypes and variants defined as DNA isolates with less than 10% sequence diversity in the L1 gene (de Villiers *et al.,* 2004).

2.2.1 Family Papillomaviridae

Papillomaviridae represents the most complex, heterogeneous and highly diverse group of human pathogenic viruses. Papillomaviruses are causative agents of usually benign tumors (warts and papillomas). The whole genomes of 189 PV types have been completely sequenced and identified (Bernard *et al*., 2010). Papillomaviruses (PVs) infect the epithelia of vertebrates as birds and most mammals including humans, where they can cause neoplasias or persist asymptomatically.

Papillomaviridae are small DNA viruses with icosahedral non-enveloped capsid (55nm). All PVs contain a circular double-stranded, DNA genomes approximately 8 kb in size and typically contain eight genes (Bernard *et al.,* 2010 and Cann, 2005). The viral DNA is associated with histone-like proteins and encapsidated by 72 capsomeres. In spite of their small size, their molecular biology is very complex (de Villiers *et. al.,* 2004). Regulatory sequences required for the viral replication and trascription are concentrated in a non coding region termed as the upstream regulatory region -URR (Fig. 2.1) (zur Hausen, 1996).

PVs genome has a long control region (LCR = 10%), an early transcription region (E = 50%) and late transcription region (L = 40%). Their genomes have up to 10 ORF and transcribed in one direction (zur Hausen, 1996). E region regulates genome persistence, DNA replication, and activation of the lytic cycle. The oncogenes, E5, E6, and E7, modulate the transformation process. E1 and E2, modulate transcription and replication (Fig. 2.2) (Munger and Howley, 2002).
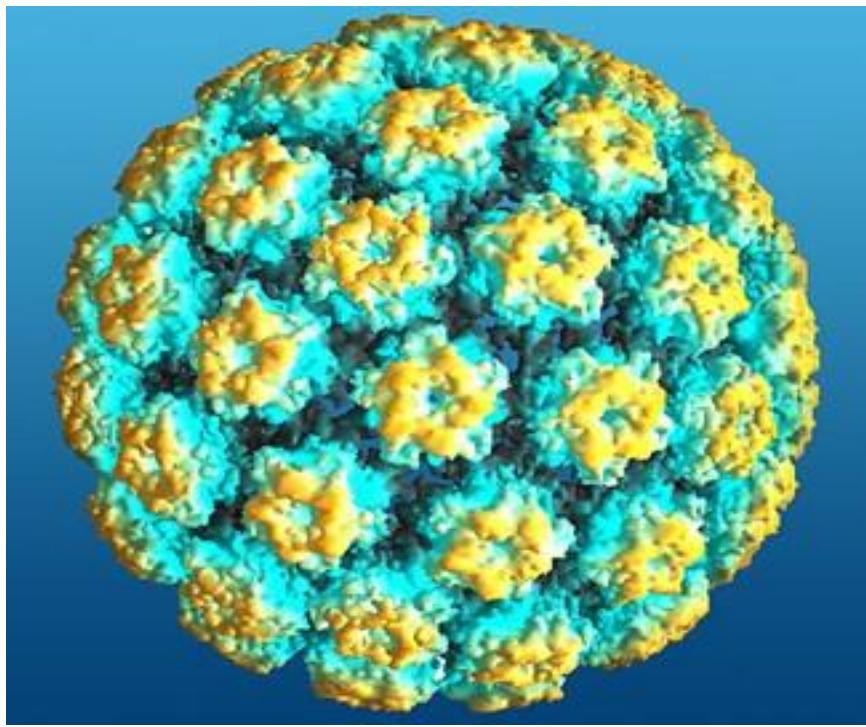
Figure 2.1   The Human Papillomavirus capsid**.**

http://virologyspring2011.wordpress.com/2011/02/28/human-papilloma-virus-a-primer/

2.2.2 Genus and species

Papillomaviruses were classified by comparison of viral genomes, due to the lack of a culture or serologic system (Bernard, *et al.,* 2010). De Villiers *et al.,* 2004   described the topology of phylogenetic trees, quantitative thresholds in nucleotide sequence comparisons and biologically distinguishing features (host species, target tissues, pathogenicity, genome organization) that determine the classification of PVs on the level of genera. Different genera share less than 60% nucleotide sequence identity in the L1 ORF. Full-length sequences of complete genomes have more than 23%, but less than 43% nucleotide sequence identity when comparing genera of the Papillomaviridae. Species within a genus share between 60% and 70% nucleotide identity (de Villiers *et. al.,* 2004).

The nomenclature of PV genera was based on the Greek alphabet (Bernard, *et al.,* 2010). Now the family Papillomaviridae, contains 29 genera formed by 189 papillomavirus (PV) types isolated from humans (120 types), nonhuman mammals (64 types) , birds (3 types) and reptiles (2 types) (Bernard *et al.,* 2010).

Human PVs were members of five genera (Alpha-, Beta-, Gamma-, Mu- and Nu-PVs), with some biological properties shared within the genera. For instance, in the Alpha-papillomavirus genus, 15 different species are found (de Villiers *et al*., 2004 and Bernard *et al.,* 2010).

2.2.3 HPV types

HPVs are large common heterogeneous group of viruses and each type is known by a number (de Villiers *et al.,* 2004). The abbreviation "HPV" was used with H standing for human or *Homo*. HPV (number) is only given after isolation and characterization of the complete genome De Villiers *et al. 2004* described the taxonomy of Human Papillomaviruses from HPV1 to HPV96. Bernard *et al*., 2010 allocated new HPVs up to HPV124.

Human papillomavirus cause a variety of benign cutaneous or mucosal warts and papillomas and cancer. Papillomaviruses are absolutely species-specific and extremely tissue specific. HPV individual viruses show tropism for either cutaneous or mucosal surfaces usually at specific sites of the body. Cutaneous HPVs contribute indirectly to skin carcinogenesis by blocking apoptosis in cells exposed to ultraviolet light and permitting the survival of UV-damaged cells (Jackson and Storey, 2000)

Mucosal HPVs affects the mucosa (cervix , mouse, throat and anus). High-risk HPVs as HPV16, HPV18 and others causing at least 90% of cancers of the cervix cancer and are also linked to more than 50% of other anogenital cancers. Presence of HPV16 and 18 increase the risk of developing cancer (zur Hausen, 1996).

2.2.4 Papillomaviruses types, subtypes and variants

PV types represent best the species. All HPV types that belong to the same species, and will presumably have properties similar or identical to the type species. New PVs identified diverged 2 to 10% from defined types. The traditional PV types within a species share between 71% and 89% nucleotide identity within the complete L1 ORF. Subtypes of PV types are defined as being gnomically 2–10% different from any PV type. Variants

showed about 1 to 2% sequence diversity (de Villiers *et. al.,* 2004). A new or novel putative PV isolate is recognized if it's complete genome has been sequenced and it's L1 sequence differs by more than 10% from the closest known PV type (de Villiers *et. al.,* 2004).

2.3 High and low risk HPVs

More than 40 HPV genotypes have been identified in ano-genital tract. HPVs can be separated into high, moderate or low risk types, depending on their biological oncogenic potentials. high- risk HPVs cause lesions that have a propensity for malignant progression, where as low-risk HPVs are associated with benign warts.

High risk HPVs are associated with cervical cancer as HPV types; 16, 18, 31, 33,35, 39, 45, 51, 52, 56, 58 and 59 . Low-risk HPVs are associated with benign epithelial proliferations such as HPV types ; 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89 (Smith *et al*., 2007). Probable carcinogenic HPVs are associated with cervical cancer as HPV types; 26, 53, 66, 68,73 and 82. Undetermined oncogenic risk as HPV types; 2a, 3, 7, 10, 27, 28, 29, 30, 32, 34, 55, 57, 62, 67, 69,71, 74, 77, 83, 84, 85, 86, 87, 90 and 91 (Smith *et al*., 2007; Simon *et al.*, 2004 and Shailja *et al*., 2008).

2.4 HPV genome

All PVs have the same genome organization and their circular dsDNA genome associate with cellular histones (Howley, 1996). PVs genome a long control region (LCR = 10%), an early transcription region (E = 50%) and late transcription region (L = 40%). PVs genomes have up to 10 ORF and transcribed in one direction. Early genes E1, E2, E4, E5, E6 and E7 encode replicative nonstructural proteins (Fig. 2.1) (zur Hausen, 1996).

The early proteins E1 and E2 are necessary for viral DNA replication and bind to the origin of replication and control transcription (Frattini and Laminis, 1994). E4 is a fusion protein and involved in the alteration of cytoskeleton network (Doorbar *et. al.,* 1991). E5 gene encode a membrane protein with a weak transforming activity (Leptak *et al.,* 1991). High risk HPVs E6 and E7 are the main cellular transforming proteins (Munger *et. al.,* 1989b; Werness *et. al.,* 1990). Recently, E7 of a low risk HPV type (HPV38) was shown to exhibit transforming activities (Calderia *et. al.,* 2003).

The late (L) region (3kb) encodes major and minor capsid proteins, and a non coding hyper variable long control region - LCR (1-kb) which regulate viral replication and gene expression (Munger *et al.*, *et al.*, 2004). L1 open reading frame (ORF) encodes the major capsid protein responsible for reactive epitopes (antigenic domains) for group type-specific neutralization.

L2 codes for an additional structural component of the viral capsid (75000 Dalton). The non-enveloped structure renders Papillomaviruses relatively resistant to heating and to organic solvents. HPVs have tropism for squamous epithelial cells. The papillomavirus life cycle is strictly linked to the epithelial differentiation program and production of virus particles is dependent on terminal cell differentiation (Bonnez *et. al.*, 1993).
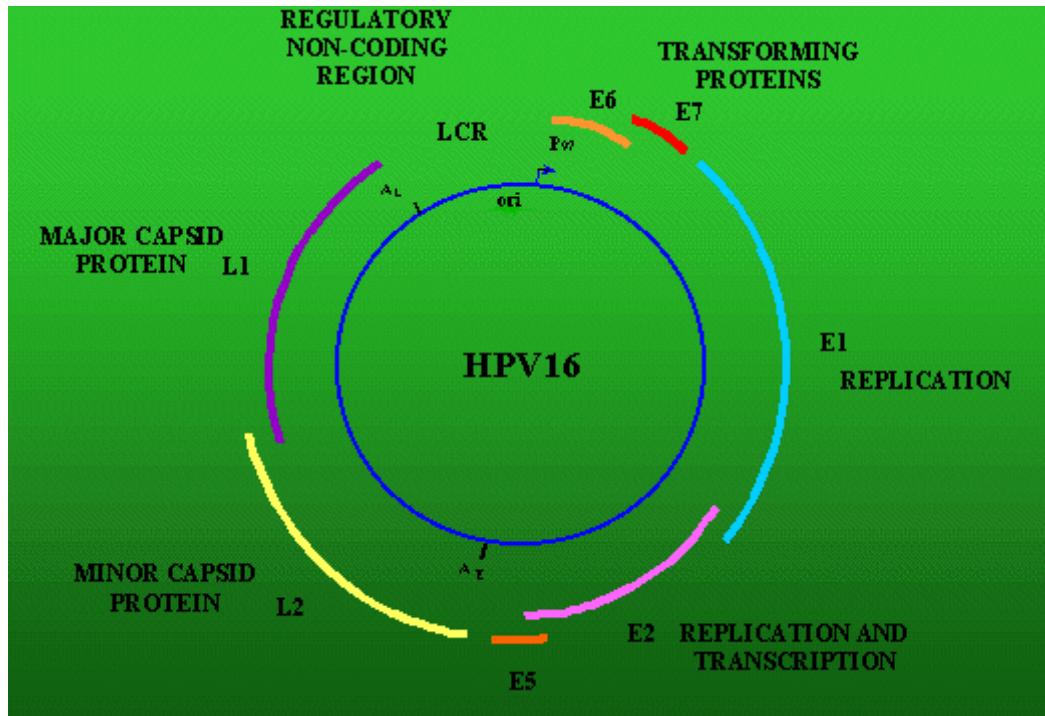
Figure 2.2   The Genome of Human Papillomavirus .

http://papillomaviridae-usf.wikispaces.com/

The virus structural proteins L1 and L2 are therefore detected only in the upper layers with differentiated cells in the infected epithelium. This property is common to all known HPVs (Schwartz, 2000).

2.4.1 E1 protein

E1 and E2 proteins are necessary for viral DNA replication and bind around the origin of replication (Frattini and Laimins, 1994). E1 codes for a polycistronic RNA, the protein has site-specific DNA binding functions, binds and hydrolyzes ATP, possesses ATP-dependent helicase activity and is essential for papillomavirus replication. It also interacts with cellular DNA polymerase. The E1 protein binding site in the origin of replication, localized in the proximal region of the LCR, represents an 18 nucleotides imperfect palindrome. Bidirectional unwinding of this region is a prerequisite for viral DNA replication. Besides L1, the E1 open reading frame represents the most conserved structure among different papillomavirus types (Fig. 2.2) (zur Hausen, 1996).

2.4.2 E2 Protein

E2 also have repressor activity on viral early promoter as a copy control mechanism (Howley and Lowy, 2001). The E2 open reading frame encodes for two to three different proteins l acting as transcription factors. They affect viral gene expression and represent major intragenomic regulators by forming dimers at specific binding sites. HPV16 and HPV 18 E2 protein function as transcriptional activators in human cervical keratinocytes. The C-terminal domain of the HPV 16 E2 gene acts as transcriptional repressor (regulator) and interferes with the activity of the full length E2 protein (Fig 2.2) (zur Hausen, 1996).

Deletion of the E2 ORF in cervical cancer facilitates cells transformation to malignant state. Mutations in E2 and in E2 DNA binding sites within the viral LCR lead to enhanced immortalizing activity of HPV 16 DNA. In cancer development, disruption of E2 appears to represent a late event. Integration of HPV16 DNA was also noted in advanced cervical intraepithelial neoplasia - CIN (zur Hausen, 1996).

1.2.4.3 E4 Protein

E4 protein originates from a viral RNA transcript formed by a single splice between the beginning of the E1 open reading frame and the E4 open reading frame. This mRNA is the major transcript in HPV-induced lesions. The E4 protein is exclusively localized within the differentiating layer of the infected epithelium. E4 protein disrupted normal differentiation and established favorable conditions for viral maturation (zur Hausen, 1996). E4 proteins associate with the keratin cytoskeleton altering its network (Doorbar *et.al.*, 1991). HPV16 E4 induces collapse of cells cytokeratin network. PV types sharing tissue specificity have limited homology in E4 DNA sequences . HPV1 E4 protein has been identified as a zinc finger protein (Fig. 2.2) (zur Hausen, 1996).

2.4.5 E5 Protein

High risk HPV E5 genes encode highly hydrophobic E5 proteins (80 amino acids). HPV-16 E5 protein is localized mainly within the endosomal membrane, Golgi apparatus, and to a lesser extent the plasma membranes (Conrad *et. al.,* 1993). Papillomas inducing HPVs E5 genes encode short hydrophobic proteins. Due to the integration of the HPV genome during malignant progression, the E5 gene is not expressed in cervical tumors and may contribute to cellular transformation by reducing the growth factor

requirements of HPV infected cells. HPV E5 gene is not necessary for the transforming activities of HPVs. HPV-16 E5 induce transformation related cellular changes in mouse fibroblast lines or mouse epidermal keratinocytes (Straight *et. al.,* 1993), augment proliferation (Storey *et. al.,* 1992) and increase cellular DNA synthesis in cooperation with EGF in human keratinocytes (Straight *et.al.,* 1993). HPV16 E5 protein complexes with a variety of trans membrane proteins. E5 ORF of oncogenic HPV types is deleted in cervical cancer. E5 may plays a role in early steps of HPV infection but not for maintenance of malignant transformation (Fig.2.3) (zur Hausen, 1996)

2.4.6 E6 oncoprotein

HPV E6 genes encode proteins of 160 amino acid in size. They are made up of two domains, which are involved in zinc binding (Grossman and Laimins,1989). Oncogenic HPV types strongly interfere with host cell cycle control mechanisms. E6 oncoprotein degrade p53 protein, that activated upon phosphorylation via DNA damage sensing proteins. Activated p53 stops the cell cycle in the G phase as a result of direct stimulation of p21cip1 by this molecule. But when major DNA damage or high amounts of viral replication occur, p53 may activate an apoptotic pathway (Shamanin *et. al.,* 1994: Bunney *et. al.,* 1992). E6 destruction of p53 renders the cells unable to prevent the accumulation of genetic mutations that may lead to cancer. E6 and E7 have counter functions that can lift the cells blocks and direct cells to enter S phase (Fig. 2.3) (Shamanin *et. al.,* 1996).

HPV16 E6 oncoprotein enter the nucleus of host cells (Le Roux, and Moroianu, 2003). immortalization of keratinocytes require the combination

of E6 and E7 (Munger *et. al.,* 1989a).   High-risk HPV E6 protein complexes with p53 and degrades it and reprogramming the cellular ubiquitin /protein ligase E6AP (Huibregtse *et. al.,* 1991).E6 proteins anti-apoptotic activities interfere with the antiproliferative functions of p53. HPV depends on the cellular DNA synthesis machinery and stimulate S-phase progression to replicate their genome, resulted in P53 over expression (Lepik and Ustav, 2000). E6 may have p53 independent transformation activities (Fig. 2.3) (Song *et al.,* 1999)

High-risk HPV E6 proteins induce rapid degradation of p53 through ubiquitin-dependent proteolysis, so HPV infected cells have lower levels of unstable p53 (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).

HPV16 E6 also attained cell transformation inducing and maintaining high levels of   human telomerase reverse transcriptase (hTert) in cervical carcinoma cells (Klingelhutz *et. al.,* 1996).   Most tumor cells maintain telomere length and telomerase activity (Bryan *et. al.,* 1997). Immortalization of keratinocytes requires activation of hTert and the p16 pathway (Dickson *et. al.,* 2000). Binding of high-risk HPV E6 proteins to PDZ proteins results in E6AP mediated ubiquitination and proteolysis (Fig. 2.3) (Gardiol *et. al.,* 1999).
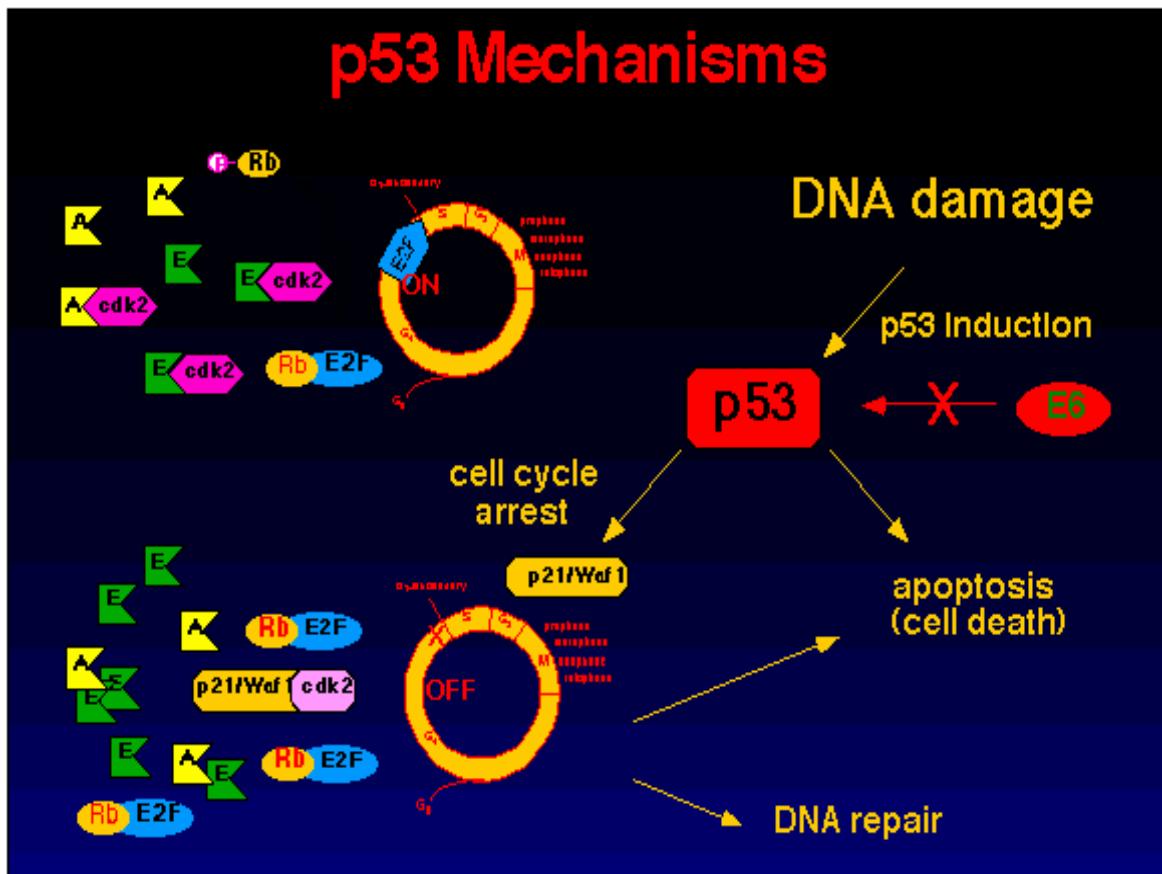
Figure 2.3 HPV E6 interactions with P^{53}

2.4.7 E7 oncoprotein

HPV E7 proteins are small nuclear phosphoproteins, rather acidic polypeptides of 100 amino acid residues. HPV E7 protein has a short half-life that rapidly degraded by proteasomal degradation (Munger *et al.,* 1989a and Munger *et al.,* 2001). The high risk E7 proteins associate with the retinoblastoma (pRb) to facilitate progression into S phase (Vousden, 1994).

E7 subvert cellular targets such as pRB are indispensable for HPV replication and the full viral life cycle (Flores *et al*., 2000 and Thomas *et al.,* 1999). A single E7 polypeptide inactivates multiple molecules of cellular target proteins HPV E7 could interfere with the ability of p53 to induce G1 growth arrest (Hickman *et al.,* 1994). HPV-16 E7 forms complexes with hypophosphorylated pRb, leading to its inactivation and permits S phase entry (Fig. 2.4)(Cobrinik *et. al.,* 1992).

E7 proteins encoded by high-risk and low-risk HPVs differ in several biochemical and biological properties. Low risk HPV-6 and -11 E7 proteins bind pRb with a lower efficiency than the high risk HPV-16 and -18 E7 proteins, but fail in targeting it for degradation and do not transform the cell (Munger *et. al.,* 1989b and Barbosa *et. al.,* 1990). Sequence comparison revealed that high risk HPV E7 have an aspartic acid residue in high-risk HPV E7 proteins (Asp 21 in HPV-16 E7) versus a glycine residue in the low-risk HPV E7 sequence Gly 22 in HPV-6 E7) (Sang and Barbosa, 1992).

HPV E7 protein degradation of pRb is necessary for its transforming activity, and induce its proteolytic degradation, decreasing the abundance of growth suppressive hypophosphorylated pRb increasing the pool of transcriptionally active E2F, independent of cdk activity, resulting in deregulated G1/S transition increasing their ability to induce DNA synthesis

and cellular proliferation (Fehrmann and Laimins, 2003). Upon phosphorylation late in G1, the hyperphosphorylated pRb does no longer interact with E2F, converting E2F into a transcriptional activator (Fig. 2.4) (zur Hausen, 1996).

E7 protein interferes with cells proliferation, differentiation and apoptosis (Flores *et al.,* 2000). E7 binding with pRB degrade it and liberates E2F transcription factors that are necessary for cell and viral replication high risk HPV E7 induce pRB proteolyses degradation and inactivation. E7 alters cellular metabolism; glycolytic processes and intracellular pH regulation. HR- HPV E7 function as a mitotic mutator by inducing centrosome abnormalities and increased cellular genomic plasticity Low levels of of E7 can inactivate pocket proteins (Fig. 2.4) (Flores *et al.,* 2000 and Munger *et al.*, 2001).

E7 carboxyl terminus is important determinant for cellular transformation wide array of nuclear and cytoplasmic proteins interact with this region of E7 E7 alters cellular metabolism including glycolytic processes and intracellular pH regulation (Munger *et al.*, 2001).

HR- HPV E7 act as mitotic mutator that rapidly induce centrosome duplication (number) resulting in multipolar and mitosis abnormalities in primary human keratinocytes and increased genomic plasticity of the host cellular genome points as well as E6. E7 may generates the host chromosomal changes (genomic instability and aneuploidy) resulted in and malignant progression (Duensing *et. al.,* 2001).

HPVs persist for many years, E6 and E7 delays keratinocytes differentiation. Many HPV positive cancers contain consistent patterns of aneuploidy, suggesting that changes in chromosome number are

important events in malignant progression. HPV E7 proteins interact with the major regulators of the cell cycle retinoblastoma tumor suppressor protein (P^{Rb}) and the related "pocket proteins" p107 and p130 (Munger *et al.*, 2004).

E7 binding with Rb mediates its degradation through the ubiquitin proteosome pathway, allows for productive replication in differentiated suprabasal cells (Munger *et al*., 2004).

HR- HPV E7 proteins associate with cyclins A and E as well as cyclin-dependent kinase (cdk) inhibitors p21 and p27 (Scott *et al*., 2006). E7 proteins bind with histone deacetylases (HDACs) which is important for the role of E7 in immortalization as well as episomal maintenance (Michelle *et al*., 2004).
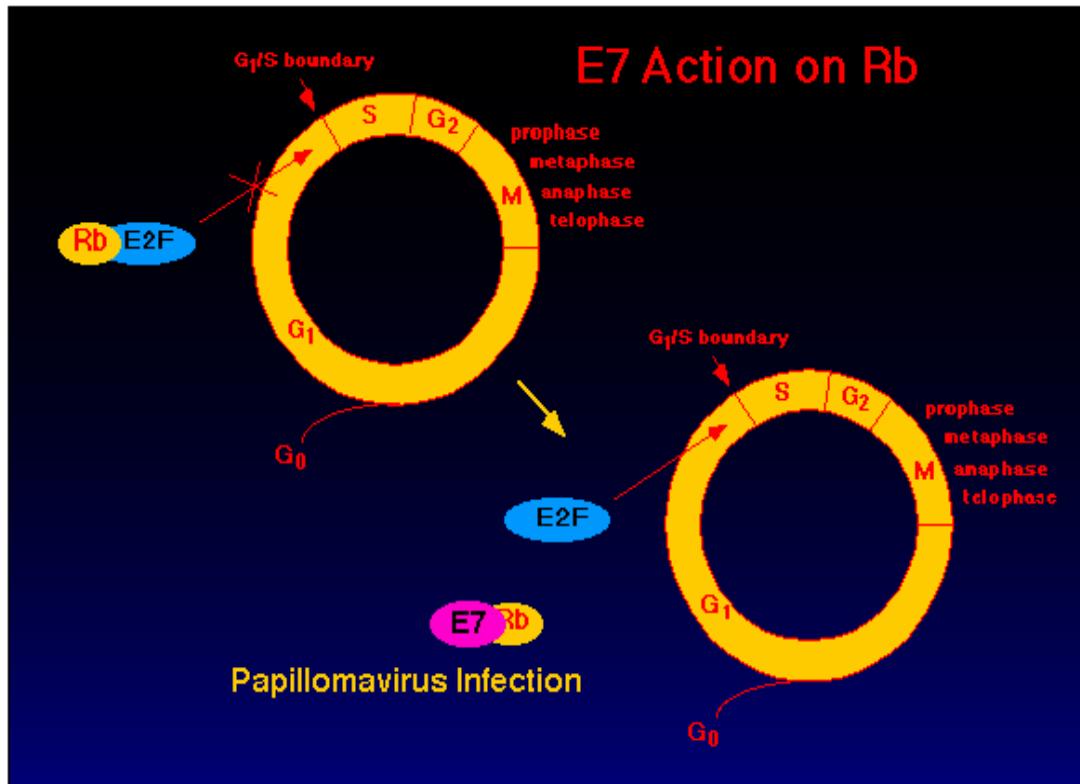
Figure 2.4   HPV E7 interactions on P^{Rb}

http://telemedicine.org/warts/cutmanhpv.htm.

2.5 HPV transmission and life cycle

HPV is sexually transmitted and there is a correlation between the number of sexual partners and the HPV prevalence as in sex workers (77%) (zur Hausen, 1996 and Mak *et. al.,* 2004). Salivary transmission by oral sex may lead to infections at oral sites by anogenital HPVs. Close skin to skin contact such as mother to baby was also suggested (Antonsson *et. al.*, 2003).

Human Papillomaviruses (HPVs) induce hyperprolifrative lesions of cutaneous and mucosal epithelia (Fehrmann and Laimins, 2003). The productive life cycle of HPV is directly linked to the epithelial cell differentiation. Following entry into keratinocytes in the basal layer, HPV genomes are established as episomal which replicate in synchrony with the cellular DNA replication (Howley and lowy, 2001).

HPV genomes maintenance is associated with expression of early HPV oncoproteins E6 and E7 and E5 and replication proteins E1 and E2. Following cell division, infected daughter cells leave the basal layer, migrate towards the suprabasal regions and begin to differentiate. In contrast to uninfected keratinocytes, which exit the cell cycle as soon as they detach from the basement membrane, HPV infected cells enter into S phase after reaching the suprabasal layer (Doobar *et. al.,* 1997). This entry into S phase results in amplification of the viral genomes to thousands of copies per cell. Concurrent with the viral DNA amplification is the synthesis of E1, E4 proteins along with capsid proteins resulting in assembly of infectious virions. Subsequently, virions are released into the environment as the upper layer of the epithelium is shed (Fehrmann and Laimins, 2003).

In cervical carcinomas, high-risk HPV genomes are integrated into the cellular host DNA that abrogates the inhibitory actions of the E2 proteins on the viral promoter of the E6 and E7 genes. This results in high level expression of E6 and E7, and most likely contributes to cellular transformation and cancer (Longworth and Laimins, 2004). The productive life cycle of HPVs is linked to epithelial differentiation. Papillomaviruses are thought to infect cells in the basal layer of stratified epithelia and establish their genomes as multicopy nuclear episomes. In these cells, viral DNA is replicated along with cellular chromosomes. Following cell division, one of the daughter cells migrates away from the basal layer and undergoes differentiation. In highly differentiated suprabasal cells, vegetative viral replication and late-gene expression are activated, resulting in the generation of progeny virions. Since virion production is restricted to differentiated cells, infected basal cells can persist for up to several decades or until the immune system clears the infection (Longworth and Laimins, 2004).

HPV E6 and E7 oncoproteins targeted P^{Rb} and P^{53}. During the viral life cycle, these proteins facilitate stable maintenance of episomes and stimulate differentiated cells to reenter the S phase. The E1 and E2 proteins act as origin recognition factors as well as regulators of early viral transcription. L1 and L2 proteins form icosahedral capsids for progeny virion generation (Longworth and Laimins, 2004).

Variations in carcinogenic potential among HPVs are principally governed by the E6 and E7 proteins; specifically by the capacities of these proteins to alter or destroy key cell cycle regulatory molecules (Critchlow and Koutsky, 1995 and Bosch, *et. al.*, 2002).

2.6 Integration of viral genome

Establishment of HPV infection is tied to the tissue proliferative activity of epithelial cells and, in the case of extensive tissue repair, the viral infection can become widely disseminated and persistent in keratinocytes. HPV DNA is frequently integrated into the host genomes in cancers, and this inactivates E2 repressor protein and allows over expression of E6 and E7, resulting in lifetime persistence of certain viral genes in the cell (Schiffman *et. al.,* 1993). High risk HPVs persist for decades undetected by the immune system as apoptosis not triggered and increasing the risk of cancer (Kashima *et. al*., 1992 and Koutsky *et. al*., 1988).

2.7 HPV and cell immortality, transformation and metastasis

In persistent HPV lesions, viral genomes in the basal cells continue to stimulate the cells to ignore the accumulated DNA damage (Gross, 1983). High risk HPVs E6 and E7 stimulated the production of clones with an extended life span that have passed mortality 1 or M1 for not immortal cells (Shamanin *et. al.,* 1996). Telomerase maintained and stabilized telomere length and capping function, allowing continuous cells division and immortalization. E6 and additional cell mutations activate and stabilize telomeres and allow cells to pass 2nd stage mortality 2 or M2 (Shamanin *et. al.,* 1996).

Chapter Three
Materials and Methods

3.1 Materials

3.1.1 Study approach and ethics

The present   study was approved by the research board of   the Sudan University for Science and Technology.   The consent to use the samples for diagnostic and research purposes was obtained from the Sudanese National Health Laboratory.

3.1. 2 Study type and design

This   retrospective study designed   to detect   HPV genotype using conventional PCR (Peltier).

3.1.3 Study area and time

The study was held during 2013 and 2014 (patient came during 2009 - 2013) at   the Department of Histopathology of the National Health Laboratory Khartoum - Sudan.

3.1.4 Study population

Paraffin   embedded sections   of   Sudanese women (n, 63)   with histologically confirmed cervical cancer (squamous cell carcinoma) with age ranges from 21 to 85 years, were selected. The controls (n, 17) were selected from married and unmarried, their age ranges from 28 to 65years. The controls with complications other than cancer and were histologically normal.

3.1.5 Samples

Each sample was handled with new glove and a specimen of 20 sections (5µm) were cut with new microtome blade each time   and collected into a sterile screw capped Eppendorf tubes.

3.1.6 Data collection

Personal and clinical data from patients and controls were recorded in special questionnaire (Appendix II).

3.1.7 Data analysis

Personal and clinical data   of controls and cervical cancer patients were statistically analyzed and linked with virological data by - SPSS12.

3.2 Methods

3.2.1 DNA extraction

3.2.1.1 Removal of paraffin by Xylene

Two ml of Xylene were added three times to screw capped Eppendorf tubes containing sections of cervical squamous cell carcinomas and control group. Then vortexed for three minutes and were left for 30 min in rotator mixer. Each sample was centrifuged   at 13200 rpm for 10minutes and the supernatant was removed leaving the tissues pellet. Xylene (1ml) was added three times to each   sample.

3.2.1.2 Addition of Ethanol

Two ml Ethanol   different concentrations (100%, 90%, 80% and 70%) were added (gradual addition of water) to each sample respectively, and vortexed very well before and after placing at rotator mixer   and centrifuged at 13200rpm for 10 minutes. Then the supernatant was removed carefully by the pipette and

immediately a new concentration of ethanol was added to each sample pellet (Appendix I).

3.2.2 DNA extraction kits

The DNA was extracted by Viral-spin R Viral DNA extraction Kit (Intron Biotechnology, South Korea) (Appendix III). This kit utilizes advanced silica-gel membrane technology for DNA isolation. Lyses buffer (500µl) was added to each sample and vortexed for 15 second. After   incubation at room temperature (10min), 20µl of Proteinase K solution (20mg/ml) was added to each sample , and incubated at 55°C for 10 min.

Then the binding buffer (700 µl) was added to each sample and mixed very well by gentle vortexing. The Spin column was fixed in a 2ml receiver (collection tubes). The lysate was loaded on the column and centrifuged (13200rpm/1min). The solution in the collection tube was discarded and the spin column was placed back in the same 2ml collection tube. The washing buffer A (500µl) added to each column and centrifuged (13.200rpm/1min.). Then washing buffer B (500µl) was added to each column and centrifuged at 13.000rpm for 1min. The solution in the collection tube was discarded and the spin columns were placed back in the same 2ml collection tubes and the tube was centrifuged (13,200rpm/1min.). The column was placed in sterile RNASE-free 1.5ml Eppendorf tube and the Elution buffer (60µl) was added directly into the center of the dry membrane. After incubation at room temperature for one minute   , then centrifuged (13,200rpm/1min). The extracted DNA was stored at -20°C until used.

3.2.3 Measuring DNA concentration

The DNA concentration was measured by automated spectrophotometer (at 260nm)**.** One microliters from each DNA sample was diluted in 999µl TE buffer (1:1000) and was shaken well, vortexed and then read to measure the DNA

concentration of each sample. The concentration of the DNA working dilution was 100ng/µl.

3.3 Polymerase chain reaction - PCR

General primers GP5+/GP6+ normally used for detection of small portion (diagnostic band 140 - 150bp) of mucosal HPV L1 Late capsid gene (Oligo Microgen- Korea) (Plate 4.1).   All DNA samples were screened by GP5/GP6 primers. All GP positive samples were retested with L1 type specific primers for HPV 16 (Plate 4.2, diagnostic band 194bp) and HPV 18 (Plate 4.3, diagnostic band 339bp) (Oligo Microgen- Korea) (Appendix V).

3.3.1 PCR Protocol: GP5+/GP6+ for detection of mucosal HPVs

Primers sequence (Appendix Va):

 GP5+/   Forward: 5´- TTTGTTACTGTGGTAGATACTAC-3´

GP6+/   Reverse   5´- GAAAAATAAACTGTAAATCATATTC-3´

(AL-Shabanah *et al*, 2013).

 PCR mix per sample:

Master mix (Appendix IV)	3 µl
primer GP5	3µl
primer GP6	3µl
H2O	11µl
Total volume	20µl
Sample DNA template	5µl
PCR reaction per sample (20µl)	
GP positive control	5µl

PCR program

Hot start	94ºC/9 min
40 cycles Denaturation	94ºC/1min
Annealing	55ºC/1min
Extension	72ºC/2min
One cycle extension	72ºC/5min
Cooling	4º C

 Agarose gel electrophoreses of the PCR products

Ten µl of the PCR products   were loaded into 2% Agarose gel . The expected product size was 140-150bp (Plate 4.1)**.**

3.3.2 PCR Protocol: Type specific for HPV 16

HPV 16 Primers sequence:(Appendix Vb).

Forward: 5´- TACCTACGACATGGGGAGGA -3´

Reverse   5´- GCAATTGCCTGGGATGTTAC -3´

(Ge *et al* ,2012).

PCR mix per sample

Master mix (Appendix IV)	3µl
HPV 16 forward primer	3µl
HPV 16 reverse primer	3µl
H2O	11µl
Total volume	20µl
DNA template	5µl

PCR reaction per sample ;

HPV16 positive control (5µl)

PCR program:

Hot start		94ºC/5 min
40 cycles	Denaturation	94ºC/1min
	Annealing	56ºC/1min
	Extension	72ºC/1min
	Elongation	72ºC/5min
	Cooling	4ºC

Gel electrophoreses of the PCR products

Ten µl of the PCR products   were loaded in 1.5% agarose. The expected diagnostic band for HPV16 type specific PCR was **194bp** (Plate 4.2).

3.3.3 PCR Protocol: Type specific for HPV 18

HPV 18 Primers sequence (Appendix Vb).

  Forward: 5′- TGGTGTTTGCTGGCATAATC -3′

  Reverse   5′- GCAGCATCCTTTTGACAGGT -3′

(Ge *et al* ,2012).

PCR mix per ample:

Master mix (Appendix IV)   3µl

HPV 18 forward primer   3µl

HPV 18 reverse primer   3µl

H2O   11µl

Total volume   20µl

Sample DNA template   5µl

PCR reaction per sample ;

HPV 18 positive control (5µl)

PCR program:

Hot start   94ºC/5 min

40 cycles   Denaturation   94ºC/1min

            Annealing   56ºC/1min

             Extension   72ºC/1min

            Elongation   72ºC/5min

            Cooling   4ºC

Gel electrophoreses of the PCR products

Ten µl of the PCR products   were loaded in 1.5% agarose gel .The expected
diagnostic band for PCR product for HPV18 was **339bp** (Plate 4.3)**.**

3.4 Agarose gel electrophoresis

Agarose gel 2% was used for GP5+/6+ (diagnostic band less than or equal 150bp), and a concentration of 1.5% was used for a diagnostic bands more than 200bp up to 600bp.Ten microliters of each PCR products was loaded on agarose gel in the tank submerged with buffer (Appendix I) and then run at 75 volt for 90 minutes. For each gel a DNA ladder marker, positive control and a negative controls and SiZer™-100 DNA Marker (Appendix VI). were loaded and then the samples. The gel then transferred into a gel documentation system, to be viewed under UV light and photographed. A PCR results were regarded as positive if it has the same size as the expected size of the diagnostic band as read by the ladder marker and in comparison to the positive control. The positive controls were used to be compared with PCR product and to insure efficiency of PCR kits. If a band appears in the lane for negative control, the experiment will be cancelled and repeated due to contamination.

3.5 Quality control

Standard procedures for preventing contamination were strictly applied. New gloves for each sample and step and clean lab coat was used. DNA samples and PCR products were kept in separate boxes. The PCR kits were divided into three separate sets (aliquots). Ice Block was used during preparation of PCR mix inside sterile lamina flow hood. Distilled water for antibiotics were used each time and were kept closed and frozen until needed. No mobiles or chat with colleagues during PCR. The condition of each experiment was written and each experiment was photographed and   the results were recorded. The bench and the hood were cleaned by ethanol before and after work and a UV light was set on overnight.

Each DNA sample and PCR product were fully labeled using water proof permanent fine tip black marker (sample number, PCR reaction and date).

Chapter Four

Result

4.1 Patients and controls

4.1.1 Patients with cervical cancer

Formalin embedded tissues blocks (n = 63) of Sudanese women with histologically confirmed cervical squamous cell carcinomas (CSCCs) were selected. Ethical clearance was obtained from the research committee of college of post graduate studies of the Sudan University for Science and Technology. Their mean and age range were 55.5 ± 14.8 years and 20 to 85 years, respectively.(Fig. 4.1) .The ages of 50.8% (32/63) of the patients were below 55years, and 49.2 % (31/63) their ages were above 56 years The patients were mostly came from central Sudan (n = 46 ; 73%), western (n = 9; 14.3%), eastern (n = 5; 7.9%), northern (n = 2; 3.2%) and southern (n = 1; 1.6%) (Fig. 4.2). Most cervical cancer patients were house wives (n = 56; 88.9%), and fewer were labors (n = 3; 4.8%), farmers (n = 2; 3.2%) and free work (n = 2; 4.2%), (Fig. 4.3).

The degree of tumor differentiation of cervical squamous cell carcinomas for these patients, were mostly moderately differentiated (n = 30; 30/63 47.6%), and 18 were poorly differentiated (n, 18 ; 18/63 28.6%) and 15 were well differentiated (n, 15 ; 15/63 23.8%), (Fig. 4.4).

4.1.2 Control sample

`   Formalin embedded tissues blocks of Sudanese women (n = 17) with benign lesions - clinical conditions other than cervical cancer (Cervical cancer) were recruited as controls. Their clinical presentations were; cervicitis   (n = 5 ; 29.4%), atrophic endometrium (n = 4 ; 23.5%), endometrial polyp (n = 4 ; 23.5%), secretary endometrium (n = 2 ; 11.7%), missed product of conception (n = 1 ; 5.9%) and simple cystic endometrial (n = 1 ; 5.9%). (Fig. 4.5).

Control's ages ranged from 20 to 65years, and their mean ages was 37 ± 8.9. Most of them their ages below 65 years (n = 16; 16/17   94.1%) and only one above 56 years (Table 4.1 and Fig. 4.6). These controls were mostly from central Sudan (n, 9 ; 9/17   52.9%), western (n, 7; 7/17   41.2%) and northern (n, 1; 1/17 5.9) (Fig. 4.7).They mostly worked as house wives (n = 14; 14/17   82.4%) and employers   (n = 3; 3/17   17.6%)(Fig. 4.8).
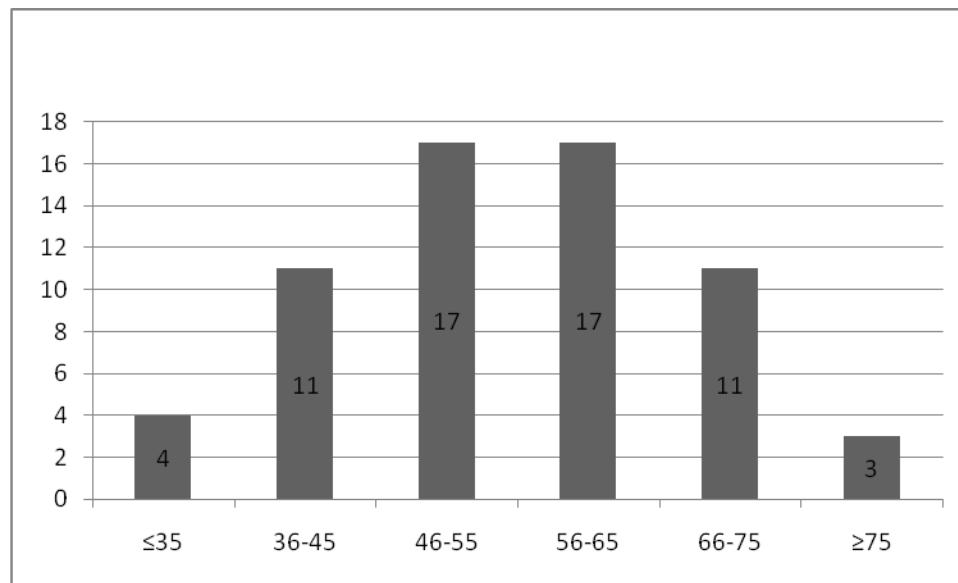
**Fig 4.1 Age group of cervical cancer patients (n, 63).**
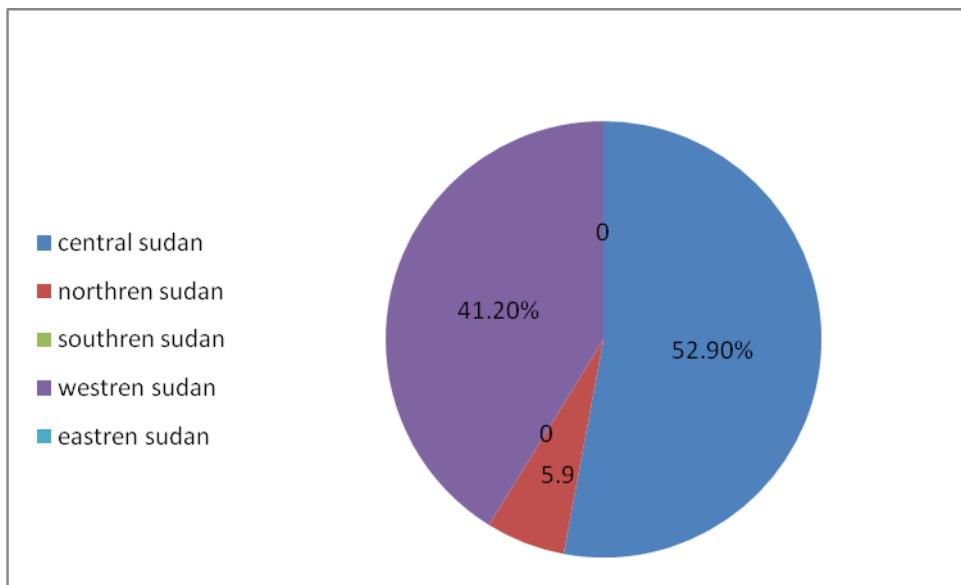
**Fig. 4.2   Geographic sites of cervical cancer patient's (n, 63)**
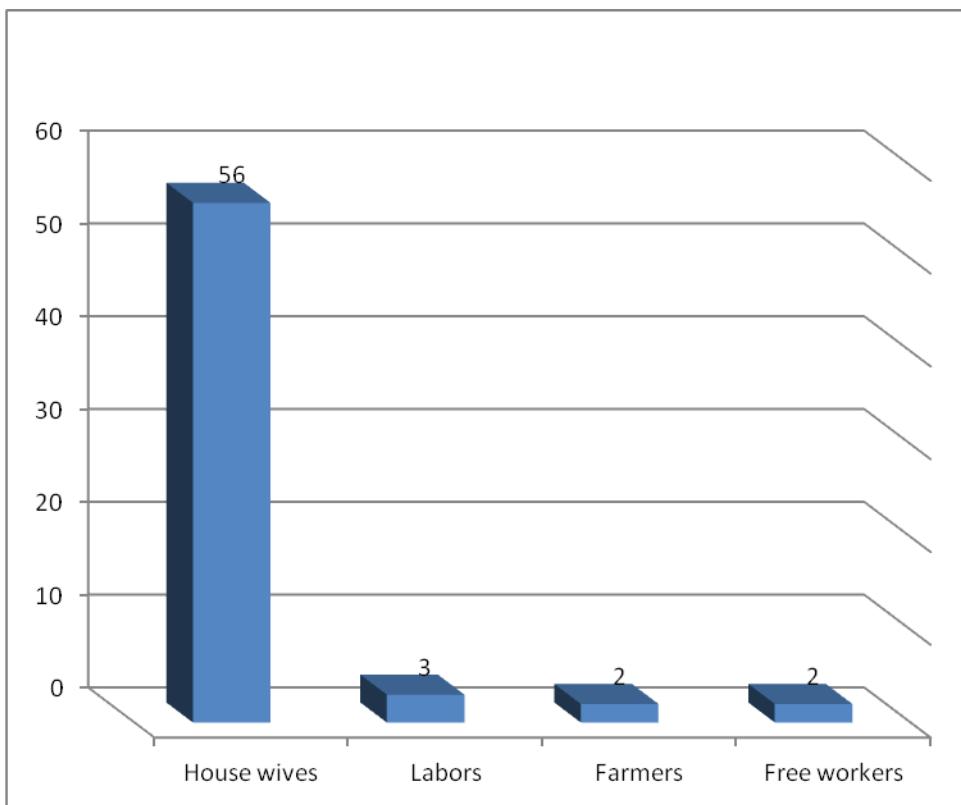
**Fig. 4.3 Jobs of   cervical cancer patients (n, 63)**
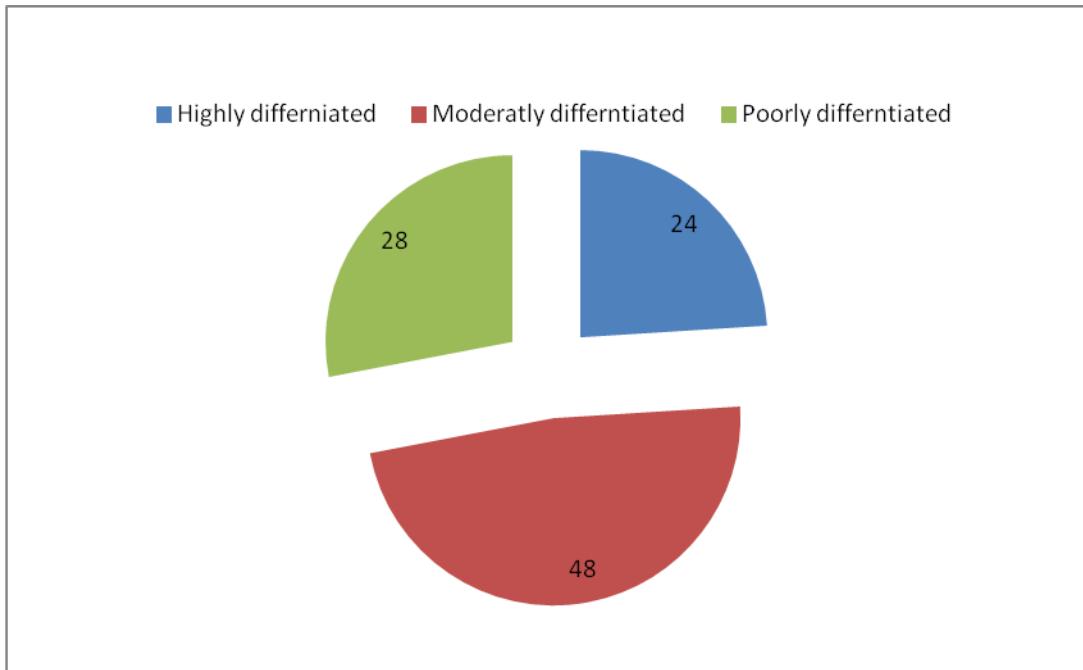
**Fig.4.4 Tumor differentiation of cervical cancer patient's (n, 63)**
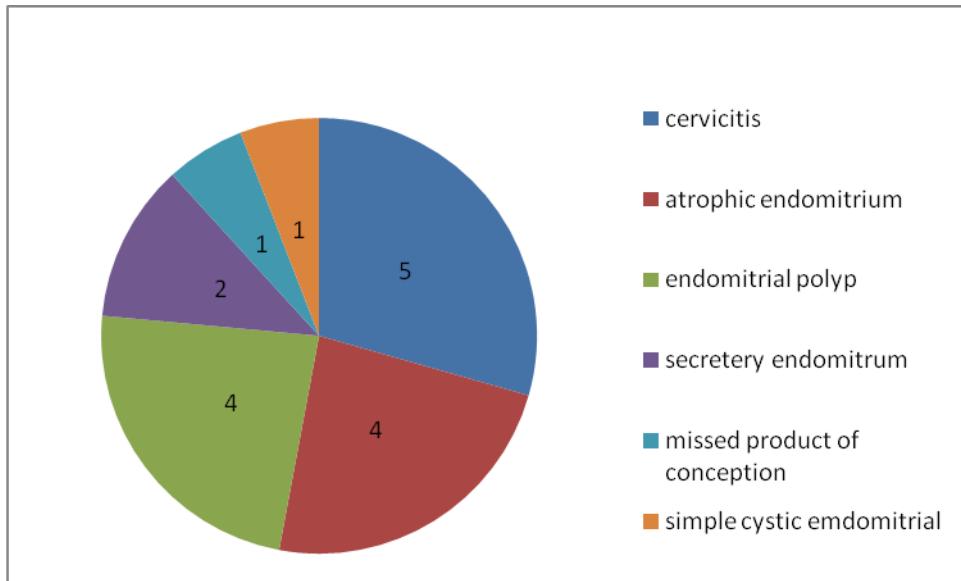
**Fig. 4.5   Control's (n, 17) clinical conditions**
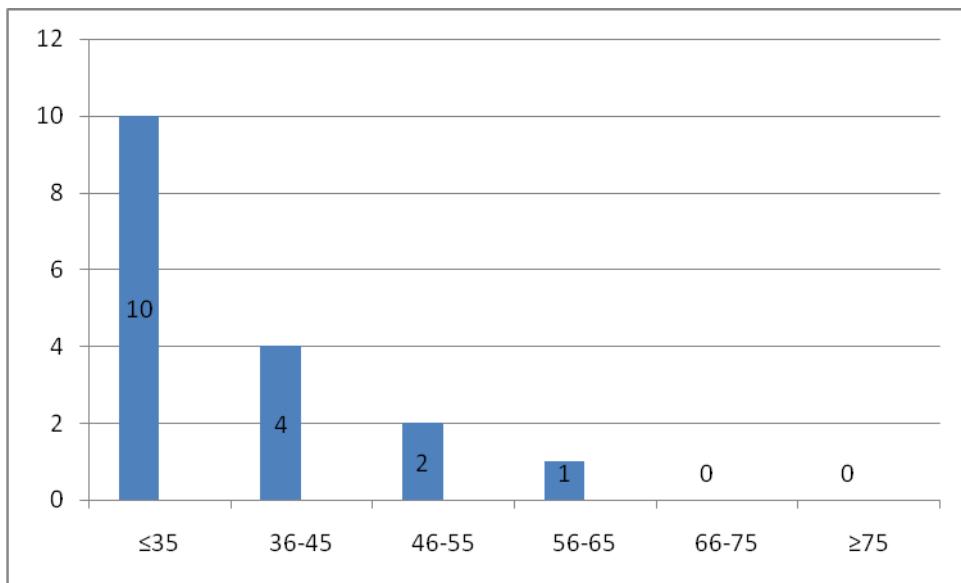
Fig. 4.6 Control's (n, 17) age groups
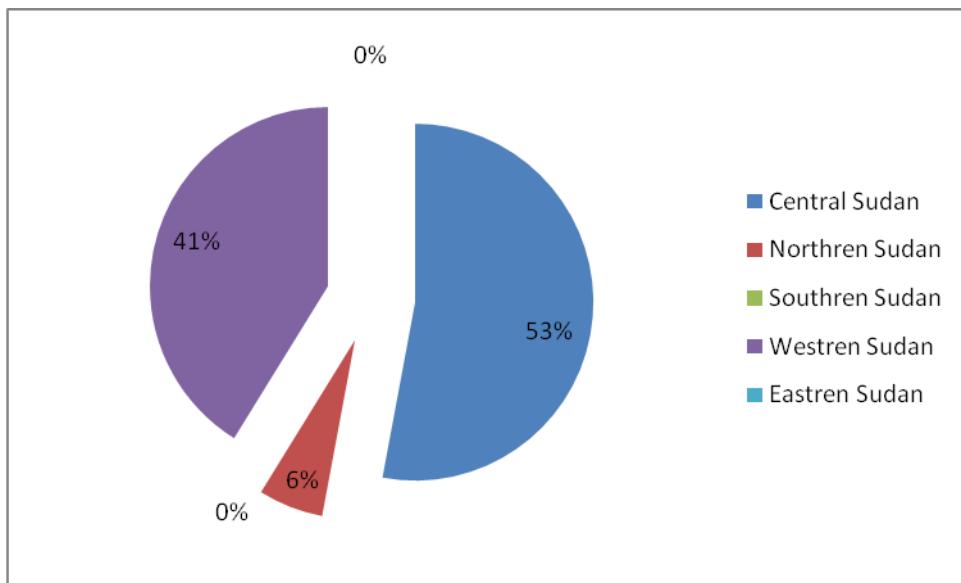
Fig. 4.7 Control's (n, 17) geographical sites in Sudan.
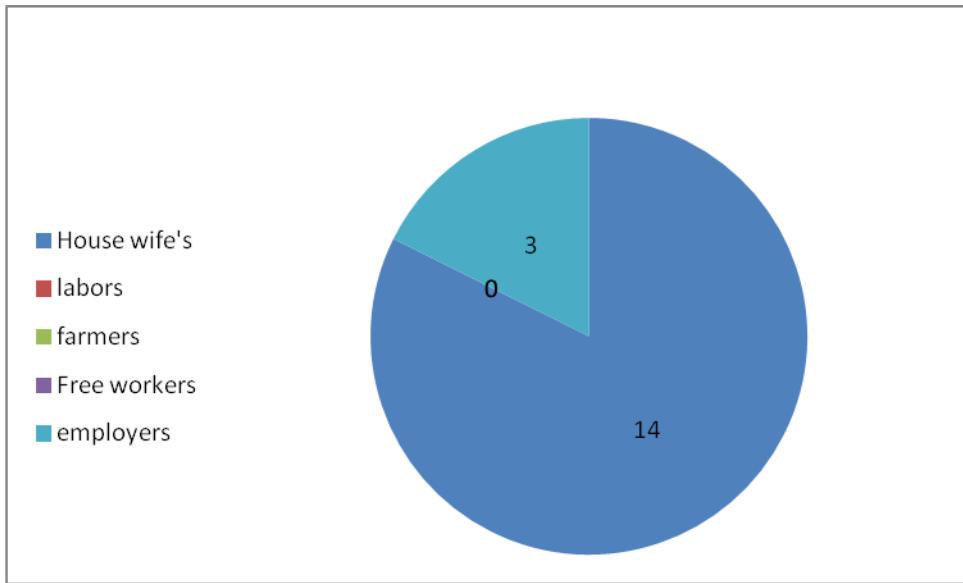
Fig. 4.8   Control's (n, 17) jobs

4.2   Detection of mucosal HPVs by PCR

4.2.1   GP5+/GP6+ PCR for cervical cancer patients and controls

The general primers GP5+/GP6+ are HPV non specific universal primers that commonly used to amplify partial constant sequences of L1 gene - late major capsid protein   for detection of mucosal HPV types. GP- PCR results indicates the presence of mucosal HPVs and didn't identify its type (Plate 4.1). Their HPV diagnostic band was 140-150bp (Plate 4.1). All cervical squamous cell carcinomas (n = 63)   and of controls (n, 17)   and were screened for   mucosal HPVs. GP5+/GP6+ primers were positive in   (n = 47 ; 47/63   75 %) patients and were negative in (n = 16 ; 16/63 25%) . All controls were HPV negative.

The DNA of all GP5+/6+ positive samples were subjected to a second round of HPV16 and HPV18 type specific PCR primers to identify HPV 16 and 18 types from the GP PCR positive samples.

3.2.2 HPV16 type specific PCR for patients

HPV16 type specific PCR primers were used to amplify and identify only HPV16 L1. The diagnostic positive band for HPV16 ranges from   190 to 200bp (Plate 4.2.). High annealing temperature was used to insure specific amplification of only   HPV type 16 L1 sequence. When HPV16 type specific primers used, HPV types other than HPV16 will not be amplified. HPV16 was detected in   (n = 33 ; 33/47 = 70.2   %) and were negative in (n = 14 ; 14/47 29.8 %)(Fig. 4.9).

4.2.3 HPV18 type specific PCR for patients

HPV18 type specific PCR primers were used to amplify, isolate and identify HPV18 L1 type specific sequence of the major capsid gene. HPV18 positive diagnostic band ranged from   330 to 340bp (Plate 4.3). HPV18 was identified in four samples (8.5   % ; 4/47) and   was negative in (91.5% ; 43/47) (Plate 4.3)(Fig. 4.9).

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 - +

Raw 1

500bp

Raw 2

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 - +
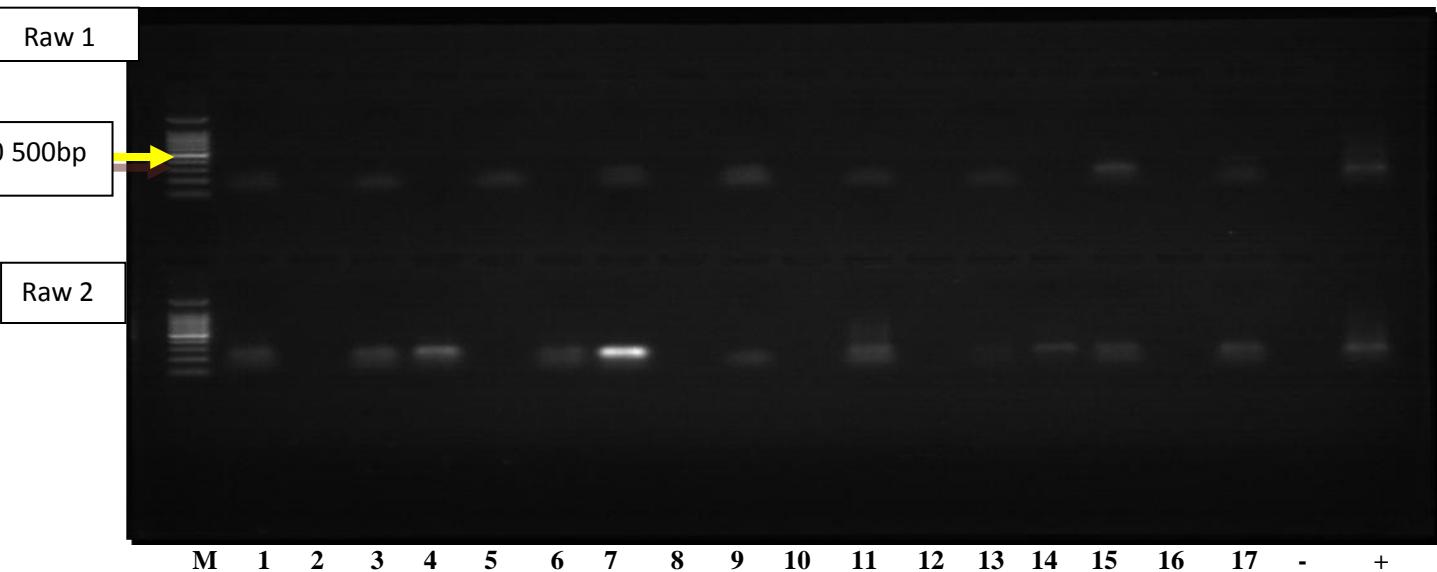
Plate.1   Mucosal   HPV GP5+/GP6+ PCR in 2% agarose gel for   Sudanese women   with   cervical   cancer (GP   diagnostic   band   140   to   150bp)

Raw one : Lane M DNA ladder molecular marker. Lane 1 to 17 GP PCR product. Positive lanes [1, 3, 5, 7, 9, 11, 13, 15 and 17]. Negative lanes [2, 4, 6,8, 10, 12, 14   and   16].   Controls;   (-)   negative   and   (+)   positive. **Raw two** : Lane M DNA ladder molecular marker. Lane 1 to 17 GP PCR product. Positive lanes [1, 3, 4, 6, 7, 9, 11, 13, 14, 15 and 17]. Negative lanes [2, 5, 8, 10, 12, and 16]. Controls; (-) negative and (+) positive.
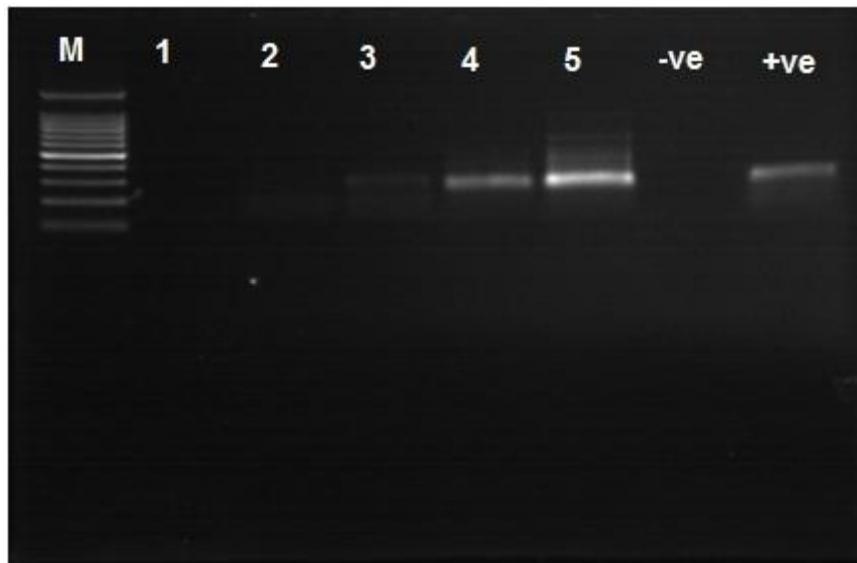
Plate 4.2 Identification of HPV 16 using type specific PCR primers in Sudanese women with cervical cancer

Agarose gel (1.5%) electrophoresis for PCR product s of HPV16 type specific primers Well M DNA ladder molecular marker, wells from 1 to 5 DNA samples of Sudanese women with cervical squamous cell carcinomas, HPV16 negatives [1, 2, & 3] positives [4 & 5], controls; (-ve) negative and (+ve) positive. HPV16 diagnostic band 190 to 200bp. .
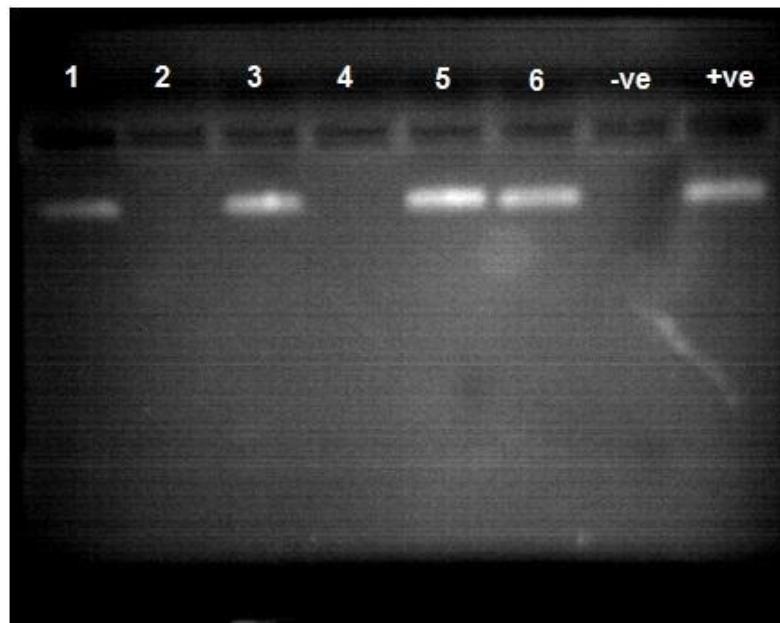
Plate 4.3   Identification of HPV18 using type specific PCR primers in cervical cancer

Agarose gel (1.5%) electrophoresis for PCR products of HPV18 type specific PCR primers. Well 1 to 6 DNA samples of Sudanese women with cervical squamous cell carcinomas Wells 1, 3, 5, & 6 were HPV18 positive.

Controls;   negative (-) & positive (+).

HPV18 diagnostic band 330 to 340bp.
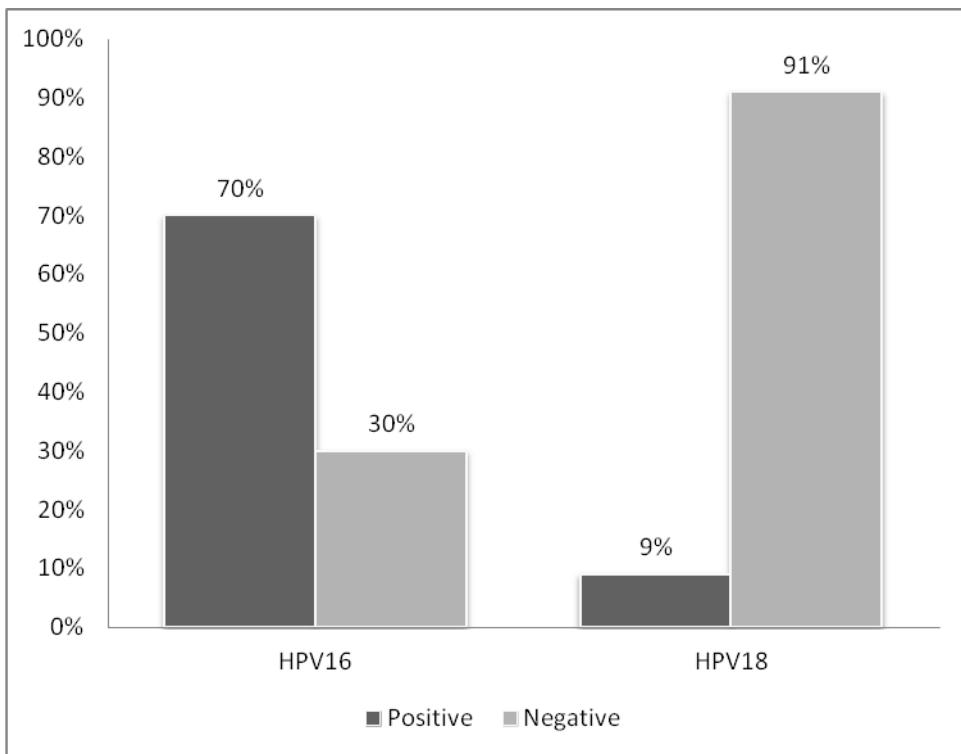
Fig. 4.9 HPV16 and HPV18 in cervical cancer patients

4.3 HPV and age

HPVs were detected at age groups 46-55years (n, 12; 19.0%), 55-56 (n, 11; 17.5%), 66-75 (n, 10; 15.9%), 36-45 (n, 9; 14.2%) < 35 years (n, 3; 4.8), and >75 years (n, 2; 2.3%). HPV was detected at ages ≤ 55years (n, 24; 38.1%), and in   (n, 23; 36.5%) in ages ≥ 56 years (P > 0.05).

HPV16 was detected at age groups 65-75 years (n = 9 ; 19.1%), 55-65 years   (n, 8; 17.1%), 36-45years (n, 7; 14.9%), 46-55years (n = 5 ;10.6%), and in (n, 3; 6.4%) at ages < 35 years and in only one case (n= 1 ; 2.1%) at ages more than 75 years. HPV16 was detected in ages ≥ 56 years (n, 18; 38.3%) than ages ≤ 55 years (n, 15; 31.9%) (P < 0.05).   .

Four   HPV18   were isolated as fellows; two (4.3%) at age 36-45years, one case (1.2%) at age 46-55 and   another one at age 65-75 years (P > 0.05). HPV18 were detected more at age ≤ 55 years (n, 3; 6.4%) than age ≥ 56 years (n, 1; 2.1%) (P > 0.05) .(Fig. 4.10).

In this study HPV16 tends to present in older ages ≥ 56 years (n, 18) than in younger ages ≤ 55years (n,15), while HPV18 tends to present in younger ages ≤ 55years (n, 3) than older ≥ 56 years ages (n, 1).
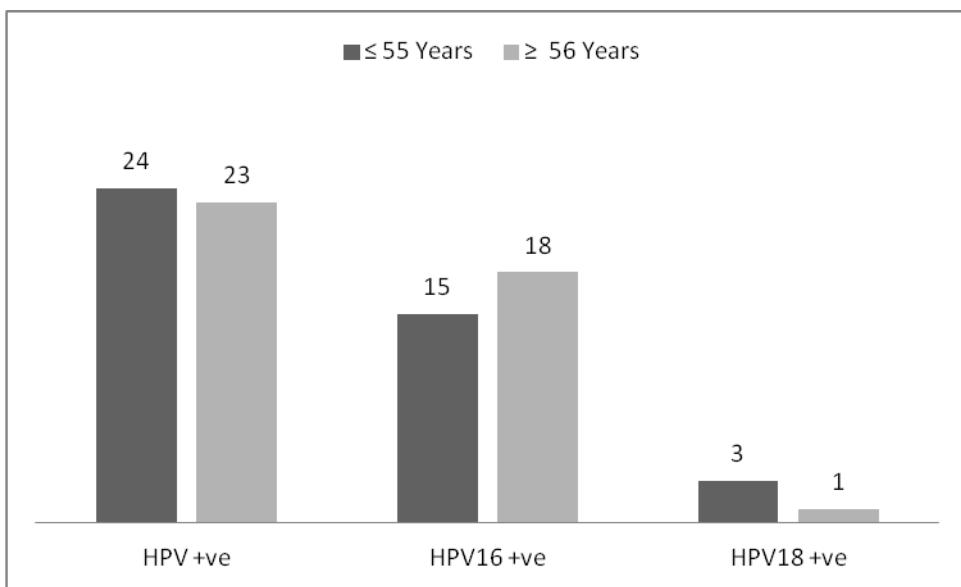
Fig. 4.10 Positivity of HPV(47/63), HPV16 (33/47), and HPV18 (4/47) in patients aged ≥ 56 and ≤ 55

4.4   HPV positivity and geographical sites

Most HPV positives were detected in patients from the Central Sudan (n, 35; 55.6%), Western (n, 8; 12.6%), Eastern (n, 2; 3.2%) Northern and Southern (n, 1; 1.6%) (P < 0.05).   .

More than half of HPV 16 positive samples (n, 26; 55.3%) were detected at the center, and less numbers from the west (n, 5; 10.6%), and only one case at north and south (n, 1; 2.1%) (P < 0.05).

Three HPV18 positives were isolated from center of the Sudan (n, 3; 6.4%) and another one (n, 1; 2.1%) from the west (P < 0.05) (Fig. 4.11).
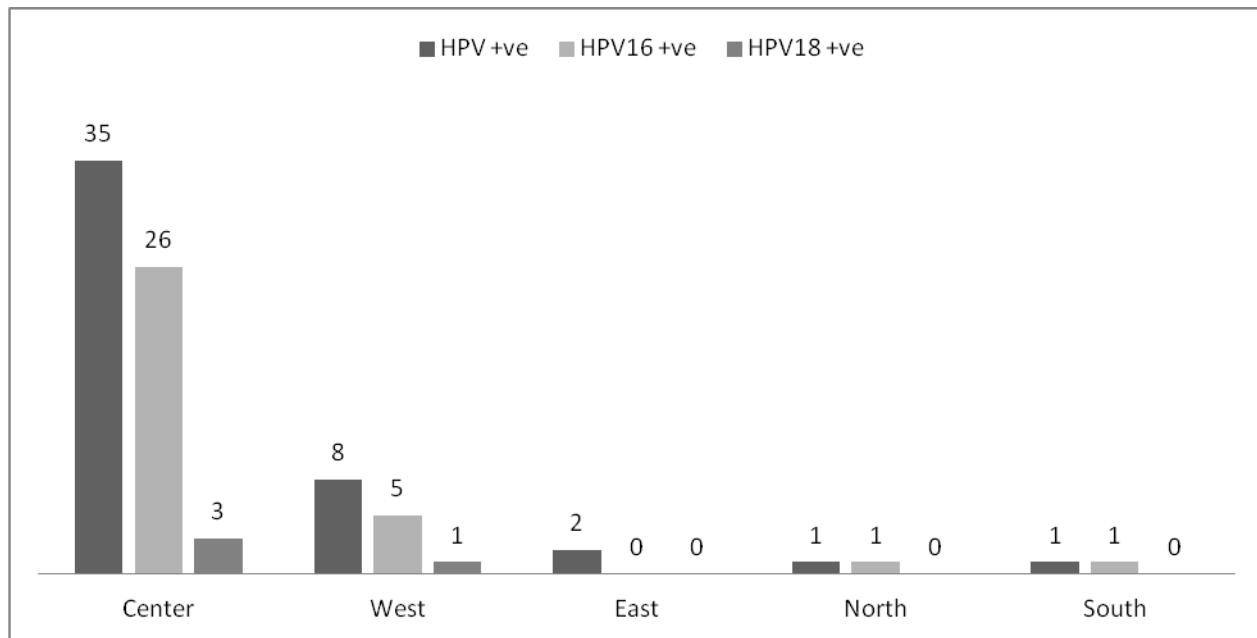
Fig. 4.11: Positivity of HPV, HPV16 and HPV18 and patient's sites

4.5 HPV and jobs

The job of most HPV positive cases were house wives (n, 40; 63.4%), then labors (n, 3; 4.8%), farmers and free workers (n, 2; 3.2 %)(P < 0.05). HPV16 was mostly identified from house wives (n, 27; 57.4%), then from labors farmers and free workers (n, 2; 4.3%) (P < 0.05). HPV 18 was identified house wives (n, 4; 8.5%) only (P < 0.05) (Fig.4.12)   .
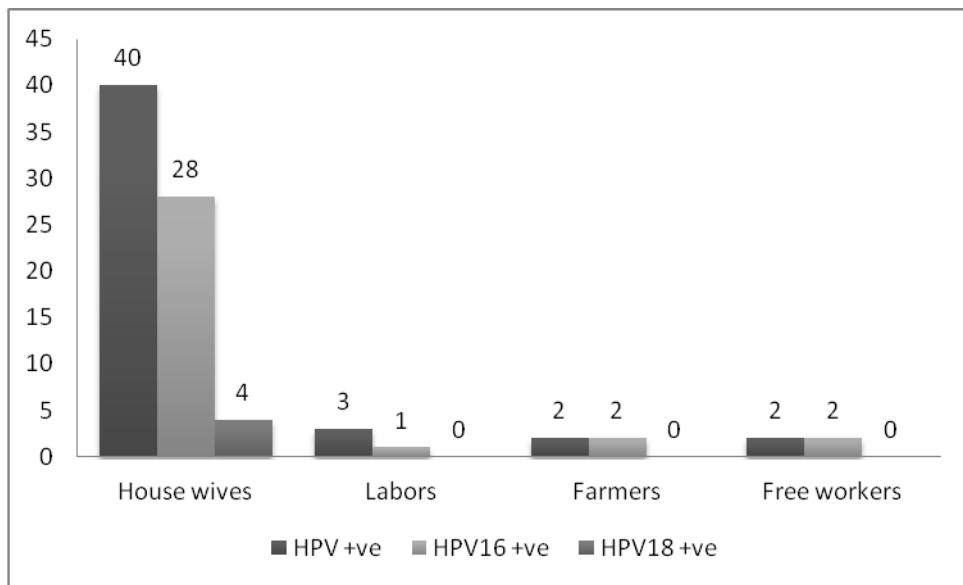
Fig 4.12   HPV and cervical cancer patient's jobs

4.6   HPV positivity and tumor differentiation

Statistically significant difference ($P < 0.05$) was obtained as most HPV positives were detected in moderately differentiated tumors (n, 25; 39.6%), in comparison to   (n, 11; 17.5%) for well differentiated and for poorly differentiated tumors. In this study HPV16 was detected in 70% (33/47) of HPV positive tumors. HPV16 present in all tumor differentiation stages. HPV16 was mostly isolated from moderately differentiated (n, 17; 36.2%) and in well differentiated (n, 9; 19.1%)   and in poorly differentiated (n, 7; 14.9%) ($P < 0.05$). Three of the four identified HPV18 were isolated from moderately differentiated tumors (n, 3; 6.4%) and   the last one isolated from well differentiated tumors (n, 1; 2.1) ($P > 0.05$) (Fig. 4.13a and Fig.4 13b).

HPV was identified from 73% (11/15) of well differentiated tumors, and from 83.3% (25/30) of moderately differentiated tumors and from 61% (11/18) of poorly differentiated tumors.   HPV16 was identified from 82% (9/11) of well differentiated tumors, and from 68% (17/25) of moderately differentiated tumors and from 64% (7/11) of poorly differentiated tumors. HPV18 was identified from 10% (1/11) of well differentiated tumors, and from   12% (3/25) of moderately differentiated tumors and from   zero% (n = 11) of poorly differentiated tumors (Fig. 4.13a, Fig 4.13b).

HPV mostly present in moderately differentiated tumors (53.2%; 25/47). HPV was detected in 39.6% of total moderately differentiated samples. Mucosal HPVs were detected in 53.2% (25/47) of   HPV +ve moderate, in well differentiated (23.4%; 11/47) and poorly differentiated (23.4%; 11/47). HPV16   was identified from 70% of all HPV positive tumor stages, and identified HPV16 in HPV +ve tumors (n = 47), in   moderately (36.2.%;17/47), well (19.1%; 9/47) and poorly (14.9%; 7/47).

HPV18 was isolated from three moderately differentiated (6.4%), and from one well differentiated (2.1%) . Ten unknown mucosal HPVs were detected by GP primers. The unknown HPVs were detected in moderately (10.4%; 5/47), poorly (8.5%; 4/47), and well differentiated (2.1%; 1/47) (Fig. 4.13a and Fig. 4.13b).
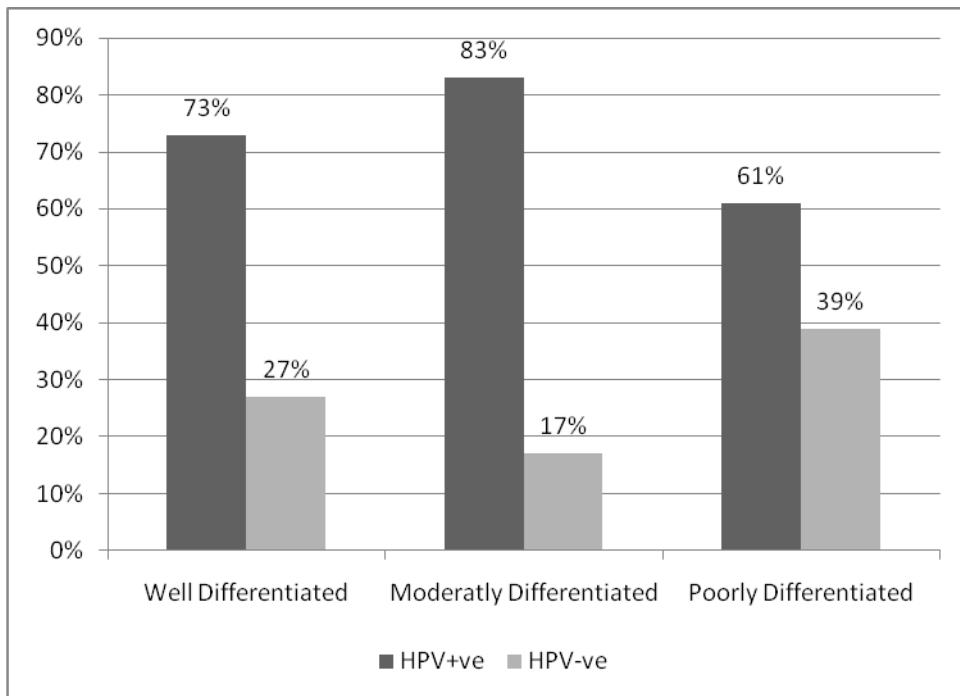
Fig. 4.13a HPV and tumor differentiation of cervical cancer patients
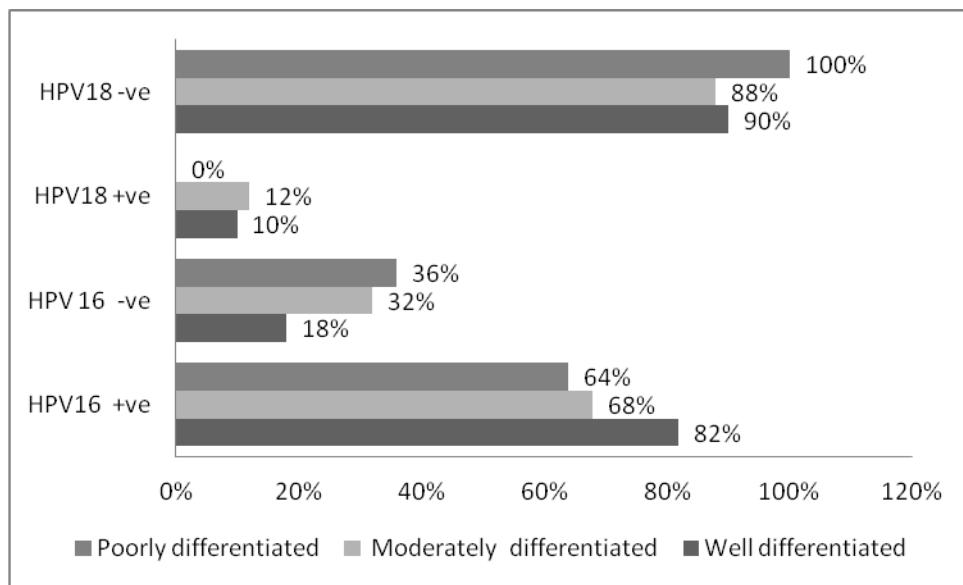
Fig. 4.13b HPV16 and HPV18 and tumor differentiation of cervical cancer patients

Chapter Five

Discussion

5.1 Discussion

Human papillomavirus is well established as a major cause of cervical cancer that leading to death in young women worldwide (zur Hausen 1982; 1996; Gissmann *et al*., 1983and Walboomers *et al* 1999). High-risk mucosal Human papilloma viruses 16 and 18 were isolated and identified as the etiological agents of cervical cancer (Boshart *et al.,* 1984 and Crum *et al.,* 1984; Muñoz *et al*., 1992 and Bosch *et al.*, 1992)). HPV16 and HPV18 were isolated from about 95% of cervical cancers (zur Hauzen, 2009). HPV 16 and 18 oncoproteins E6 and E7 were confirmed to transform cervical cells by degradation of tumor suppressor proteins P^{53} and P^{Rb}, repectively (Werness *et al.,* 1990; Dyson *et al.*, 1992; zur Hausen, 1996, 1999; 2000 and 2009).

Contradictory researches on HPV in cervical in Sudan were published (Salih *et al.,* 2010; Elasbali *et al.,* 2012 and Eltahir *et al.,* 2012). This study aimed to screen HPV, HPV16 and HPV18 in paraffin embedded sections of Sudanese women with cervical cancer. Also to explore the link between HPVs with personal (age and site) and clinical data (Tumor differentiation).

HPV

In this study HPV was detected by GP5+/Gp6+ in 75% (47/63) of cervical cancers, and all controls were negative. Lower HPV positivity 40% (16/50) in samples of cervical cancers from Sudan were obtained by Elasbali *et al.,* 2012. Higher HPV positivity 93.6 % (73/78) among Sudanese cervical cancer patients was reported by Eltahir *et al.,* 2012. In this study HPV was not detected in

controls, however, Eltahir *et al.,* 2012 in Sudan detected HPV 8.3% (3/36) of controls. The results of this study were comparable to those reported from some Arabic countries such as;   Kingdom Saudi Arabia (82%) and   Egypt (86%), (Alsbeih *et al.,* 2013; Abd El-Azim and Lotfy, 2011).

Different higher HPV positivities were reported from different countries. HPV positivity of the present study (75%) was less than that reported before. Higher HPVs positivities were reported from Kingdom Saudi Arabia (82%), Egypt (86%), Sir Lanka (90%) ,   South Africa (97%) and 100% by Walboomers *et al,* 1999 (Alsbeih *et al.,* 2013; Abd El-Azim and Lotfy, 2011; Karunaratne *et. al.* 2014 and Richter *et al.,* 2008). Almost the real ranges of HPV positivities were reported in two   meta analysis studies; 83 to 89% by Clifford *et al* ., 2003 (85 studies and 10000 patients and 90% by Smith *et al.,* 2007.

In the present study, 70% (33/47) of the HPV types identified were HPV16 (70% = 33/47) and HPV18 (9% = 4/47). HPV16 and 18 were responsible for about 80% of HPV positive cervical cancers in Sudan. The remaining 20% (10 HPVs) may be for those not identified in this study (HPV; 31, 33 and 35) (de Oliveira *et al.,* 2013).

For HPV negative results this may have been due to absence of HPV DNA in the carcinoma cells or a false-negative PCR result due to integration of HPV DNA in the cervical carcinoma which may have disrupted PCR primer target sequences or resulted in loss of the L1 ORF. HPVs may have hit and escape phenomenon.   In this study paraffin embedded sections were used and fixation process may degraded integrated DNA that lower the sensitivity of PCR and gave some false negatives.   PCR may detected only DNA of   viruses with capsid which   known to be resistant to organic solvents.   However, HPVs were detected in 99% of cervical fresh tissues (de Oliveira *et al.,* 2013). Usually very

low viral load in these cases may give false negatives as invisible DNA signals or bands. Different methods might have variable results.

In contrast to Oliveira *et al.,* 2013 who detected more HPVs in older patients (> 50 years), this research detected HPVs in all ages. This may suggest more virulent oncogenic HPV variants in Sudan that may induces cervical cancer in younger women. However, comparison of their tumor suppressor genes and HPV oncogenes sequences with others from the world may give more acceptable explanations. However, Eltahir *et al.*, 2012, who studied loss of heterozygosity and mutations in P^{53} and P^{Rb} of cervical cancer patients, did not find such associations in Sudan.

HPV 16

This study identified HPV16 in 70% (33/47) of HPV positive samples which is less than reports from; Sudan (83%), and in Ethiopia (91%) (Abate *et al* ., 2013). Less HPV positivities were reported from Sudan (2.2%, 50%), Iran (26%) and Egypt (50%) (Salih *et al.,* 2010; Elasbali *et al.,* 2012; Farjadian *et al.,* 2003, and Abd El-Azim & Lotfy, 2011). A meta analytic study reported an average HPV16 positivity of 55% (Smith *et al.,* 2007). Another meta analytic study estimates different HPV16 positivities in Asia (46%) and in North America and Australia (63%) (Cillford *et al.,* 2003). This might indicates different prevalences for HPV16 and HPV18 from continent to continent. Many authors indicated the presence of HPV16 variants, that may have various clinical outcomes and age linkages (Wentzensen *et al.,* 2013, and Zuna *et al.* 2011).

Our findings, that HPV16 was the most frequent HPV type in cervical cancer (70%), agree with other authors from; Sudan (83%), Saudia (71%), Sirlanka (67.3%), and the World (Abate *et al* ., 2013; Alsbeih *et al.,* 2013 and

Karunaratne *et al.,* 2014; zur Hausen, 1987and Bosch *et al.,* 2002). zur Hausen, 1987 showed that HPV16 DNA in cervical cancer present as episomal and not integrated and present in 50 to 80% of cervical cancers. Accordingly, HPV 16 is an stronger carcinogen and important determinant for the development of cervical cancer.

HPV18

The present study identified four HPV18 isolates (9%), which is similar to reports from; Egypt (10%) and Sir Lanka (9.2%) (Abd El-Azim and Lotfy, 2011 and Karunaratne *et al.,* 2014) . Less numbers of HPV18 out of HPV +ves were isolated before from Sudan (Abate *et al.,* 2013 and Elasbali *et al.,* 2012) and Saudia (4%) (Alsbeih *et al.,* 2013). In agreement to other authors, this research confirmed that HPV18 was less frequent and not the major carcinogen for cervical cancer (zur Hausen, 1987 and 2000).

HPV and tumor differentiation

In the present study, 47.6% of the tumors were moderately differentiated, 28.6% were poorly differentiated and 23.8% were well differentiated. Almost the same findings were reported before from Sudan (50%, 26% and 24%) by Eltahir *et al.,* 2012.

As in this study a similar tumor differentiation of cervical cancer patients was reported from Sudan; moderate (47%), poorly (29%) and well (24%)(Eltahir *et al.,* 2012). This study agree with other authors who   detected mucosal HPVs and HPV16 in all stages of tumor differentiations (zur Hausen, 1987; Clifford *et al.,* 2003, and Smith *et al.,* 2007). HPV was detected mostly in 39.6% of total moderately differentiated samples and in 53.3% of HPV +ve moderate, then well

differentiated and poorly differentiated (n, 11; 23.4%). This research isolated HPV16 from 70% of all HPV positive tumors, and identified HPV16 in HPV +ve tumors (n = 47), in moderately (36.2.%), well (19.1%) and poorly (14.9%).

HPV18 was isolated from three moderately differentiated (6.4%), and from one well differentiated (2.1%) . This study detected ten mucosal HPVs by GP primers, their types were unknown as HPV16 and 18 type specific primers were used. These unidentified HPVs were detected in moderately (10.4%), poorly (8.5%), and well differentiated (2.1%). Our results showed that HPV mostly present in moderately differentiated tumors, and HPV16 present in all tumors. Also this study noticed that the unidentified HPV present also in poorly differentiated tumor , so these viruses may have different clinical outcome or virulent as HPV16. This study suggested that HPVs may required in early stages of tumor differentiation than late stages as poorly. This study confirmed the major role of HPV16 as strong carcinogenic virus causing cervical cancer in Sudan. Young Sudanese women has to be vaccinated against HPV16 specifically. The link between HPV and clinical outcome as tumor progression and differentiation, has to be clarified with an equal numbers of tumor differentiation stages.

HPV and age

Age is very important determinant for cancer and HPV infections. Age determines risk factor prevalence habits, smoking, marital status, exposure to high risk HPVs and the differential impact of specific HPV types, onset of sexual activities, lifetime number of sexual partners, parity, Multiparity, hormonal contraceptives using, menopausal status, virus prevention methods (condom), education and vaccination (Gargano *et al.,* 2011). Cancer is multistep disease that developed overtime, and normally cancer developed in ages above 55 years. Oncogenic HPV viruses require another factors so virus- cell interactions are very

important. for younger ages may have inherited cancer mutations in their oncogenes or tumor suppressor genes (P^{53} and P^{Rb}) (Gargano *et al.,* 2011 and Porras *et al.* 2009).

In contrast to Gargano, *et al.,* 2011 and Porras *et al.* 2009, who found that HPV16 strongly associated with younger ages, the present study isolated more HPV16 from older ages ≥ 56 years (n, 18). This study identified more HPV18 from younger ages. Ethnic variations and multiparty may explain our findings. Porras *et al.,*2009 reported that 80% of cervical intraepithelial neoplasma - CIN3+ were associated with HPV 16 among young women ages 18-26 years compared with only 32% among women older than 55 years This study and other authors identified HPV16 from all ages, and it is important alarm for cervical cancer regardless to age (Gargano, *et al.,* 2011 and Porras *et al.* 2009). From the present research and as reported before it seems different HPV types may infect different age groups (Munoz *et al*., 2012).

HPV infection is most common in sexually active young women, 18 to 30 years of age and its prevalence sharply decreased after 30 years of age (Munoz *et al*., 2012). However, cervical cancer is more common in women older than 35 years, suggesting infection at a younger age and slow progression to cancer. Persistence of infection is more common with the high-risk oncogenic HPV types and is an important determinant in the development of cervical cancer (Munoz *et al*., 2012).

HPV and geographic sites

de Oliveira *et al.,* (2013) in Prasil attributed differences of HPV infections and types in two different geographic site to different ethnic origins and characteristics and the as patterns of sexual behavior. This study agree with other authors from

Sudan, that cancer patients were mostly from northern sites (central Sudan) and tribes (Idris *et al.,* 1994 and 1995). It is widely accepted that each continent have different HPV types and variants that have different biological and oncogenic pthogenicities (Bruni *et al.,* 2010 and Karunaratne *et al.* 2014).

General remarks

Reliable serological tools are not available and HPV culture is not possible, so molecular techniques are the only possible reliable methods for diagnosis and identification of HPVs (Dillner 1999).Various molecular techniques such PCR primers and probes have different DNA target sequence with variant annealing temperatures, have different sensitivities and specificities. Different results might be due to sample size and storage conditions (fresh or frozen tissues or paraffin embedded), viral load, researcher experience. Always, new reagents for DNA extraction (DNA quality), staining and new PCR kits produce strongly viable bands. PCR regents has to be divided into aliquots and keeping reagents in ice pox during preparation.

L1 region is the most conserved part of HPV genome that targeted by many primers for detection of different HPV genotypes . Universal consensus or general PCR GP5+/GP6+ primers amplify a broad spectrum of HPV genotypes (de Roda Husman *et al.,* 1995). Type specific primers designed to amplify exclusively a single HPV genotype, multiple type-specific PCR reactions must be performed separately for the same sample. This method is laborious, expensive (Molijn *et al.,* 2005). However, multiplex PCR with type specific probes may be economic. HPV general primers with low annealing temperature, and a second round of HPV PCR with high risk HPV type specific primers for GP PCR products (positive or negative). Using HPV type specific primers detected only targeted types and leave other HPV types that may produce false negatives. For more accurate sensitive

results , the whole GP positive PCR product precipitated and their bands cut and purified from the gel and cloned before using type specific primers. Commonly used   HPV16 and 18 PCR type specific primers were designed from European variants and isolates not African ones and accordingly less sensitivities resulted (Wentzensen *et al.,* 2013 and Zuna *et al.* 2011).

Conclusion

1- A total of 63 sample from Sudanese women with cervical cancer were tested.

2- HPV was detected in 47 (75%) out of the tested 63 cervical squamous cell carcinomas.

3-HPV 16 were detected in 33(70%) of the HPV positive cases while HPV 16 was detected in 4(8.5%) ,and unknown HPV type detected in 10(21%).

3-High HPV prevalence in which most of them were at age younger age ≤55 years.
4-Most of the HPV was detected at patient came from central Sudan .

5- HPVs were mostly detected in moderately differentiated tumors.

Recommendations

The present study recommend:

1- Increase the society awareness about the risk of HPV transmission and cervical cancer and the importance of regular pap test, HPV screening ,and vaccination,

2-Vaccination of Sudanese younger women(10 to 25 years) against HPV16 and 18.

3- Further studies to identify other HPV genotypes with fresh samples using multiplex PCR with   mucosal HPV multi probes.

4- The positive and negative GP PCR products, has to be rescreened with HPV type specific primers or multiplex PCR.

5- Identification of HPVs in positives samples with HPVs other than 16 and 18.

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Appendices

Appendix I

Preparations of Solutions

(1) Ethanol

100 ml	250 ml	500 ml	
90 ml + 10 H_2O	225 ml +25 H_2O	450 ml + 50 H_2O	90%
80 ml + 20 H_2O	200 ml + 50 H_2O	400 ml + 100 H_2O	80%
70 ml + 30 H_2O	175 ml + 75 H_2O	350 ml + 150 H_2O	70%

(2)10X Tris EDTA buffer(TBE buffer)   500 ml   250ml

EDTA trisidium 1.15g	2.3 g
boric acid 13.75g	27.5g
Trise base 27.25g	54.5g

adjust PH to 8.3 by NaOH.

Complete the volume of. DW to 250 ml   or 500 ml

Autoclave.

1XTBE buffer=10ml 10XTBE buffer+90ml DW..

0.5X TBE buffer =50ml 10X TBE buffer +50ml DW.

Lab indication

For preparation of stock solution start with minimum volume of water of 100 ml

and start to adjust the PH after dissolve prepare the following before start

- Autoclaved DW.

- Beaker

- Measuring cylinder

- Pyrex bottle

- Magnetic sterror

- Seal tap

* Write your name, Lab. number, Ingredient PH and date on each bottle.

* For autoclave write your name & Lab number in the bottle cap.

Appendix II

Questionnaire

Sudan University of Science and Technology
College of Graduate Studies

Sample Block No:..................................*

Age

Residence: site   City........................

Center -

North   -

West   -

East   -

Job:..

Tribe:..

Education:..............................

Awareness of the disease and cancer:..........................

Economic level:....................................

Income per year:...................................

Where do you live........... ....:

Religion:..

Habits:

a- Smoke   b- Smokeless

 tobacco dipping :a-yes   b-no   (Toombak) other...............................

Marital status:

married   - unmarried   -divorced-

-separated   - Widowed   -

NO of pregnancy:

No of abortions: *

Use of contraceptive:*

yes-

No-

unknown

If yes, the type :................................. -

Use of hormonal therapy:

yes   No   unknown

Use of condoms:

-Yes   -No   -unknown

History of other diseases:

Infectious : a- Gonorrhea   b- Syphilis   c- Herpes

Non infectious :a- Diabetes   b- Blood pressure   c- Thyroids   d- Other

Histological diagnosis :

Tumor site:............................ Tumor size:...............................

Tumor type: a- Squamous cell carcinoma b- CIN 1   C- CINII   D- CINII

 Tumor differentiation:

a- Poorly   b - Moderately   c- Highly

Tumor Grade :

a-   (I)   b- (II)   c- (III)   d- (IV)