DETECTION OF HUMAN PAPILLOMAVIRUS 16 AND 18 DNA USING TYPE SPECIFIC POLYMERASE CHAIN REACTION IN CERVICAL CANCER BIOPSES IN SUDAN

By

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Dedication

This work is dedicated

To

My parents, family and friends
ACKNOWLEDGEMENTS

First of all, I render grateful thanks to Allah for offering me assistance, health and patience to complete this work.

No words would rightfully express my deep gratitude to my supervisor Prof Abdelmelik Ibrahim Khalafalla, Faculty of Veterinary Medicine, University of Khartoum. I would like to thank him for his continuous support and encouragement. My deep gratitude is also to Prof. Mohamed Elsanousi Mohamed Elsanousi. Faculty of Medicine, University of Gezira for his supervision, advice, and unlimited support. I would like to thank Dr. Zahir Abass Hilmi who has provided me with knowledge, advice and support.

My sincerest gratitude goes to Dr. Nagla Gasmelseed, Head Department of Molecular Biology (INMO) In Gezira state in the Sudan, and Almutaz Abbas Alemam for their unlimited support and encouragement.

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The help of my colleagues is gratefully acknowledged
**List of Abbreviations**

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<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
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<tr>
<td>INMO</td>
<td>Nuclear Medicine and Oncology</td>
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<tr>
<td>Pap</td>
<td>Papanicolaou</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>HHV-8</td>
<td>Human Herpes Virus type 8</td>
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<tr>
<td>HTLV-1</td>
<td>Human T-Lymphotropic retrovirus type 1</td>
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<td>HIV-I</td>
<td>Human Immunodeficiency Viruses type 1</td>
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<tr>
<td>HIV-II</td>
<td>Human Immunodeficiency Viruses type 2</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frames</td>
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<tr>
<td>URR</td>
<td>Upstream Regulatory Region</td>
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<tr>
<td>LCR</td>
<td>Long Control Region</td>
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<tr>
<td>E</td>
<td>Early Region</td>
</tr>
<tr>
<td>L</td>
<td>Late Region</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor Suppressor Protein</td>
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<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
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<tr>
<td>VLPs</td>
<td>Virus- Like Particles</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Neoplasia</td>
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<tr>
<td>hTert</td>
<td>human Telomerase reverse transcriptase</td>
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<tr>
<td>HR-HPVs</td>
<td>High Risk – Human Papillomaviruses</td>
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<tr>
<td>E2F-1</td>
<td>cellular transcription factor</td>
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Abstract

A retrospective molecular analysis for Human papillomavirus types 16 and 18 was done on 40 formalin- fixed paraffin- embedded cervical biopsies, previously collected from women in the Central Sudan and histopathologically diagnosed as cervical cancer. According to the age, the women were divided into two groups ≤ 44 were 30% and ≥ 45 were 70%. DNA was extracted using DNA extraction kit. Type specific polymerase chain reaction (PCR) kit that contained primers for HPV16 together with primers for HPV18 was used to detect Human papillomavirus types 16 and 18 and to determine the prevalence of each type and also to determine the association of these HPVs DNA to various grades of cervical carcinoma.

PCR results showed that HPV16 and 18 were detected in 14 (35%) of the study specimens. HPV16 was detected in 57.1% (8/14) and HPV 18 was detected in 28.6% (4/14). Mixed infections were detected in 14.3% (2/14) of positive samples, Accordingly, HPV 16 is the most common HPV type associated with cervical carcinoma in Central Sudan. The HPVs were detected in 10 individuals whose ages were ≥ 45 and in 4 individuals whose ages ≤ 44. Therefore, cervical cancer is more common in woman older than 45 years, suggesting infection at a younger age and slow progressing to cancer.

An interesting finding in this study was the relatively higher rate of HPV infection in the early stage of cancer. This finding reveals the importance of the use of HPV detection as screening tool for early detection of cervical cancer.

It is recommended that a wide population-based epidemiological study be conducted to define the exact picture of this disease and to define types of HPV that are found in other parts of the Sudan. The use of suitable vaccine targeting these types will have substantial impact on cervical cancer control in the Sudan.
**الخلاصة**

لتحديد أسباب الإصابة بسرطان القولون في السودان، تم تجميع وتحليل بيانات مصورة تشمل 16 نمط سرطاني. وجدت النتائج أن 40% من النماذج كانت ذات بيئة الأورام، مع وجود زيادة في نسبة الأورام ذات الوراثة. وضعت النتائج المذكورة في دراسة تمثلية، حيث وُجد أن نسبة الأورام ذات الوراثة كانت 45%.

وبالنسبة لمستوى الضغط الهرموني، وجدت النتائج أن نسبة الأورام ذات الوراثة كانت 45%.

استخدمت دراسة أخرى استخدمت نتائج PCR لتحديد النماذج السرطانية، حيث وُجدت النتائج أن نسبة الأورام ذات الوراثة كانت 45%.

استخدمت دراسة أخرى استخدمت نتائج PCR لتحديد النماذج السرطانية، حيث وُجدت النتائج أن نسبة الأورام ذات الوراثة كانت 45%.

وأخيراً، استخدمت دراسة أخرى استخدمت نتائج PCR لتحديد النماذج السرطانية، حيث وُجدت النتائج أن نسبة الأورام ذات الوراثة كانت 45%.
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CHAPTER ONE
INTRODUCTION

Cervical cancer is the growth of abnormal cells on the surface of a woman's cervix. Most cervical cancers begin with microscopic changes in the cells located in the outer layer of the cervix. These changes, known as dysplasia, may develop into cancer. The most common types of cervical cancer is squamous cell carcinoma.

Cervical cancer represents the second most frequent malignant tumor of women worldwide with an estimated frequency of approximately 440,000 new cases per year, corresponding to about 5.8% of the global cancer incidence.

Human papillomaviruses (HPVs) are now recognized as the major cause of cervical cancer. HPVs are a group of more than 200 viruses. They are called papillomaviruses because certain types may cause warts, or papillomas, which are benign (non-cancerous) tumors. The HPVs that cause the common warts which grow on hands and feet are different from those that cause growths in the throat or genital area. Some types of HPV are associated with certain types of cancer. These are called “high-risk” oncogenic or carcinogenic HPVs. Human papillomaviruses types 16 and 18 are responsible for about 70% of cervical cancer (Munoz, 2006).

More than 50% of vulvar, penile, perianal and anal cancers, in all likelihood more than 20% of oral, laryngeal and nasal cancers contain predominantly anogenital high risk HPV types, this leads almost 10% of the worldwide cancer burden linked to these infections (zur Hausen, 1996).
In 2006, an estimated 10,000 women in the United States were diagnosed with this type of cancer and nearly 40% died from it. Cervical cancer strikes nearly half a million women each year worldwide (American Cancer Society, 2006). Lower rate of cervical cancer in the United States is attributable to the success of the widespread use of the Papanicolaou (Pap) test, which detects changes in cervical tissue, and is a major tool in screening for early identification of cervical cancer. When detected early, cervical cancer is highly treatable.

Africa as a region has one of the highest incidence rates in the world and five of the seven countries with the highest rate globally are in eastern or southern Africa (Drain, Holmes, Hughes and Koutsky, 2002).

In the Sudan cervical cancer is the second most common cancer observed among women (Globocan, 2002). The rates of cervical cancer in the Sudan was estimated as <16.8 per 100,000 woman. There are no data on the prevalence of human papillomavirus in Sudanese women with cervical carcinoma. In the present study the prevalence of HPV 16 and 18 was investigated in archival material of cervical carcinoma kept at the Department of histopathology, Medical Laboratory, University of Gezira. DNA was isolated from formalin fixed, paraffin embedded tissue, histologically classified according to the degree of differentiated cells to well, moderately and poorly differentiated squamous cell carcinoma.

The aim of this cross sectional study is to identify which HPV 16 and 18 are the causative agent for cervical cancer in the Sudan, and to provide base line information for eventual preventive measures including vaccine trials and other control activities.
OBJECTIVES

1. General Objectives:

   To detect HPV 16 and 18 in cervical cancer in the Sudan. And to determine the frequency and distribution of HPV 16 and 18 in cervical cancer biopsy samples (paraffin embedded tissue blocks) previously collected from women in Central Sudan.

2. Specific Objectives:

   To investigate the association of HPV 16 and 18 DNA to various grades of cervical carcinoma and their relation or association with age.
CHAPTER TWO

LITERATURE REVIEW

2. Human Papilloma Virus (HPV):

2. 1. Biological agents and viruses in human cancer:

Surprisingly, widespread chronic diseases, previously thought to be due to metabolic imbalances or genetic modifications, are increasingly linked to infectious events. Approximately 20% of global cancer incidence is aetiologically related to specific infections. About 5% for Bacteria (Helicobacter pylori) and helminthes (Schistosoma, Opistochoris, and Clonorchis) contribute to the development of gastric, bladder and rectal cancers and cholangiocarcinomas (zur Hausen, 2001). Viruses, however, emerge as major causal cancer factors and about 15% can be linked to tumor viruses. In addition to hepatocellular carcinoma linked to hepatitis B and C virus infections (HBV and HCV); specific types of papillomaviruses have been shown to cause a major human cancer, cancer of the cervix. They have also been implicated to in a number of other anogenital, oropharyngeal, and cutaneous cancers. Epstein-Barr virus (EBV), human herpes virus type 8 (HHV-8), and human T-lymphotropic retrovirus type 1 (HTLV-1) represent additional identified human tumor viruses (zur Hausen, 2001).

There exist good reasons to argue that still other virus types are involved in human cancers: Human immunodeficiency viruses type HIV-I and HIV-II induce severe immunosuppression and facilitate cancer induction by other persisting infections, in particular by HHV-8, Epstein-Barr virus and human papillomaviruses. Thus these agents contribute indirectly to human carcinogenesis. However, specific cutaneous papillomavirus infections 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).

It is likely that epidemiological studies may not point to the existence of as yet unknown additional tumor viruses if these are ubiquitous. Nevertheless, the mere presence of viral DNA within a human tumor represent a hint but clearly not proof for an a etiological relationship (zur Hausen, 1999).

2.2 HPV and Cervical Cancer:

Epidemiologic, cytopathologic, virologic and histologic data have established a strong association of HPV infection with carcinoma of the cervix (Munoz, Bosch and de Sanjosé, 2003).

The prevalence of HPV in cervical squamous cell carcinoma reported was between 70%-98% (Farjadian, Asadi, Doroudchi, Dehaghani, Tabei, Kumar, and Ghaderi, 2003, Stanczuk, Kay, Allan, Chirara, Tswana, Bergstrom, Sibanda, and Williamson, 2003 and Munoz et al., 2006).

Some types of HPV are referred to as “low-risk” viruses because they rarely develop into cancer. HPV types that are more likely to lead to the development of cancer are referred to as “high-risk.” Both high-risk and low-risk types of HPV can cause the growth of abnormal cells, but generally only the high-risk types of HPV may lead to cancer. Sexually transmitted, high-risk HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and possibly a few others. These high-risk types of HPV cause growths that are usually flat and nearly invisible, as compared with the warts caused by types HPV–6 and HPV–11. It is important to note, however, that the majority of high-risk HPV infections go away on
their own and do not cause cancer (Walboomers, Jacobs and Manos, 1999).

HPV 16, 18, and a number of additional HPV types have been found in about 70% of all biopsies derived from cancer of the cervix throughout the world, the remaining 30% being due to other high-risk HPV types (such as HPV-31, -33, -35, -39, -45, -51, -66). (Gissmann and Gross 1986) showed that HPV 16 and 18 are found in a substantial number of invasively growing squamous cell carcinomas of the cervix, suggesting an etiologic involvement of these viruses in development of cervical cancer.

HPV16 is the most frequently found virus type and accounts for 50 to 60% of all positive data, the presence of HPV18 vary between 10 and 20%. HPV45, a type relatively closely related to HPV18, thus cross-hybridizations between HPV45 and 18 may have led to an overestimation of HPV 18 positivity. Many of these types have been found in exceptional cases only. The mere presence of HPV DNA in the vast majority of biopsies from cancer of the cervix does not prove an etiological involvement. Early data pointing to a role derived from experimental findings were reviewed by zur Hausen (1996).

Although HPV related cancers are more common in women, increasing numbers of HPV related carcinomas of anal mucosa are being reported among homosexual men. A third group at risk for severe HPV infection is neonates. At birth, HPV can infect the mucosa of pharynx and cause large wart like lesions that can obstruct the airway (Mahdavi and Monk, 2005).
The evidence linking HPV and carcinoma of cervix has come from detection of viral genomes in neoplastic and pre-neoplastic tissues. It is now well established that HPV infection is the central causal factor for cervical cancer. So the most important risk factor for cervical cancer is infection by the human papillomavirus (HPV). Doctors believe that women must have been infected by this virus before they will develop cervical cancer.

An individual is at greater risk of becoming infected with HPV if he or she has had multiple sexual partners at any time or is the partner of someone who has had multiple sexual partners. Sexual activity at an early age also places an individual at increased risk as does a history of other sexual diseases in an individual or sexual partners. In addition to sexual activity, age is an important determinant of risk of HPV infection (Adam, Berkova and Daxnerova, 2000).

At least 50 percent of sexually active men and women acquire genital HPV infection at some point in their lives. By age 50, at least 80 percent of women will have acquired genital HPV infection (Ho GYF, Bierman, Beardsley, Chang and Burk, 1998).

Studies suggest that whether a woman develops cervical cancer depends on a variety of factors acting together with high-risk HPVs. The factors that may increase the risk of cervical cancer in women with HPV infection include number of sexual partners, younger age at first intercourse, increasing parity, younger age at first full-term pregnancy, increasing duration of oral contraceptive use and smoking.

HPV infections can occur in both male and female genital areas that are covered or protected by a latex condom, as well as in areas that are not covered. Although the effect of condoms in preventing HPV
infection is unknown, condom use has been associated with a lower rate of cervical cancer (Thomas, Ray, Koetsawang, Kiviat, Kuypers, Qin, Ashley and Koetsawang, 2001).

2.3 The HPV Structure and Genome:

Current data support the existence of more than 200 HPV genotypes. The entire genomes of about 100 HPV types have been isolated and completely sequenced (Bernard, 2005). These viruses can be classified into mucosal and cutaneous HPVs. Within each of these HPV groups, individual viruses are designated high risk or low risk according to the propensity for malignant progression of the lesions that they cause (Munoz et al., 2003).

The diameter of papilloma virus (HPV) particles amounts to approximately 55nm. PVs have circular double-stranded DNA genome with sizes close to 8kb. 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, Fauquet, Broker, Bernard and zur Hausen, 2004).

The genome of all HPV types consist of closed-circular double-stranded DNA (Figure.1). It is containing approximately 8 open reading frames (ORF) which are transcribed as polycistronic messages from a single DNA strand. The structure of the viral genome reveals remarkable similarities between different members of this virus group: generally only one strand is transcriptionally active, therefore transcription occurs in one direction only, and the localization of open reading frames reveals a remarkable degree of correspondence (Donos, Katinka and Yaniy, 1982). Sequences required for the viral replication and transcription are concentrated in a non-coding region termed as the upstream regulatory region (URR) (zur Hausen, 1996).

Papillomaviruses genome can be divided into three regions: a long control region (LCR) covering about 10% of the genome, and early (E)
and a late (L) region. The L genes codes for structural proteins, the E region mainly with regulatory functions concerned with persistence, DNA replication and activation of the lytic cycle. In short, E1 and E2 are the two viral proteins that are required for viral DNA replication, together with the host cell DNA replication machinery, E4 and E5 proteins are needed for amplification of the viral genome in the upper layers of the epithelium. The E6 and E7 proteins of high-risk HPV types are oncogenic. They cooperate to immortalize cells and also induce genomic instability. E6 and E7 abrogate the activity of the cellular tumor suppressor proteins p53 and Rb, respectively. E6 also increases telomerase activity (Huibregtse and Beaudenon, 1996).

Gissmann and Schwarz (1986) noticed that papillomaviruses expressed parts of the early region (E) of their genome in cell lines of genital cancer. They concluded that HPV might be involved in inducing and/or maintaining the transformed phenotype of cancer cells.

Infection with high-risk HPV types interferes with the function of cell proteins and also with the expression of cellular gene products. Microarray (gene chip) analysis of cells infected with HPV-31 has shown that 178 genes are up regulated and 150 genes are down regulated by HPV (Chang and Laimins, 2000).

### 2.4 HPV Oncogenes and their interaction

#### 2.4.1 E6 Oncoprotein

The E6 oncoprotein of high-risk human papillomavirus type 16 (HPV16) plays a role in the cellular transformation process. HPV16 E6 oncoproteins enter the nucleus of host cells via multiple pathways (Le Roux and Moroianu, 2003). Efficient immortalization of keratinocytes requires the combination of E6 and E7 (Munger, Phelps, Bubb, Howley and Schlegel, 1989a).
E6 proteins exert their functions by interacting with cellular proteins. High-risk HPV encoded E6 protein that forms a complex with p53 leading to functional inactivation (Werness, Levine and Howley, 1990). High-risk HPV E6 proteins have anti-apoptotic activities and can interfere with the anti proliferative functions of p53. HPV depends on the cellular DNA synthesis machinery and must stimulate S-phase progression to replicate their genome, resulted in P53 over expression which represents a major impairment for viral replication.

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

Independent of p53 HPV16 E6 was reported to activate telomerase through up-regulation of hTERT, the catalytic subunit of telomerase, which is a critical step in cellular transformation (Klingelhutz, Foster and McDougall, 1996). Activation of telomerase is closely associated with progression of CIN and cervical carcinoma, and telomerase might be a useful marker for cervical carcinoma (Leng, Bian, Sun, Fan and Chen, 2002).
2.4.2 E7 Oncoprotein:

HPV E7 protein binding and degradation of the retinoblastoma protein (Rb) are necessary for its transforming activity. High-risk HPV E7 proteins interact with pRb and induce its proteolytic degradation.

The E7 protein together with E6 provide the major transforming activities of HPVs. Expression of the E7 protein in the absence of other viral gene products leads to the transformation of rodent fibroblasts. While E7 alone can immortalize human keratinocytes, the presence of E6 greatly enhances the frequency at which this can occur (Hubbert, Demers and Galloway, 1999).

HPV-16 E7 forms complexes with hypophosphorylated Rb, leading to its inactivation and permits S phase entry (Cobrinik, Dowdy, Hinds, Mittnacht and Weinberg, 1992). HPV E7 abrogates Rb mediated regulation of the S transition of the cell division cycle. Rb binds E2F-1 and actively represses transcription from promoters containing E2F-1 sites. Upon phosphorylation late in G1, the hyperphosphorylated Rb does no longer interact with E2F-1, converting E2F-1 into a transcriptional activator. High-risk HPV E7 proteins can interact with Rb and induce its proteolytic degradation, decreasing the abundance of growth suppressive hypophosphorylated pRb increasing the pool of transcriptionally active E2F-1 (zur Hausen, 1996).

Destabilization of the Rb tumor suppressor and stabilization of p53 contribute to HPV16 E7 induced apoptosis and the ability of E7 to induce cellular transformation (Gonzalez, Stremlau, He, Basile and Munger, 2001). Binding of E7 to hypophosphorylated Rb prevents it from binding to E2F-1 and thereby promoting cell cycle progression allowing for productive replication of HPV genes.
HPV-16 E7 plays a major role in inducing centrosome related mitotic disturbances. Abnormal centrosome duplication induced by HPV E7 rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell (Duensing, Crum, Munger and Duensing, 2001a). This activity is, therefore, likely to be functionally relevant to the contribution of high-risk HPVs to malignant progression. E7 of HPV 38 was shown to have in vitro transforming activities (Calderia, Filotico, Accardi, Zehbe, Franceschi and Tommasino, 2004).
Figure 1: HPV 16 genome
2.5 HPV Life Cycle and Transmission:

Human papilloma viruses (HPVs) induce hyper proliferative lesions of cutaneous and mucosal epithelium (Fehrmann and Laimins, 2003). The productive life cycle of HPV is directly linked to the epithelial cell differentiation. Infection by papilloma viruses is believed to occur through micro traumas in the epithelium, exposing the basal cells to enter by viruses (Howley and Lowy, 2001). Following entry into keratinocytes in the basal layer, HPV replicates as the basal cells differentiate and progress to the surface of the epithelium. In the basal layers, viral replication is considered to be non productive and the virus establishes itself as a low-copy-number episome by using the host DNA replication machinery to synthesize its DNA on average once per cell cycle (Flores, 1997). In the differentiated keratinocytes of the suprabasal layers of the epithelium, the virus switches to a rolling-cycle mode of DNA replication, amplifies its DNA to high copy number, synthesizes capsid proteins and causes viral assembly to occur (Flores, 1999). Since HPVs encode only 8 to 10 proteins, they must utilize host cell factors to regulate viral transcription and replication.

In low grade cervical lesions, the HPV genomes are found exclusively as episomal DNA molecules. In contrast, in cervical carcinomas, high-risk HPV genomes are integrated into the cellular host DNA (Storey, Greenfield, Banks, Pim, Crook, Crawford and Stanley, 1992). Durst et al (1987) detected the integration of papillomavirus sequences near cellular oncogenes in some cervical carcinomas.

Integration of HPV DNA has been suggested to be an important event in the development of cervical cancer, since this often results in a disruption of the E2 ORF. Integration thus abrogates the inhibitory action of the E2 protein 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 that eventually results in cancer (Longworth and Laimins, 2004).

The E6 and E7 genes encode viral oncoproteins that target p53 and Rb respectively during the viral life cycle; these proteins facilitate stable maintenance of episomes and stimulate differentiated cells to reenter the S phase. The E1 and E2 proteins act as origin recognition factors as well as regulators of early viral transcription. The functions of the E5 and E1-E4 proteins are still unknown but these proteins have been implicated in modulating late viral functions. The L1 and L2 proteins form icosahedral capsids for progeny virion generation. The characterization of the cellular targets of these viral proteins and the mechanisms regulating the differentiation-dependent viral life cycle remain active areas for study of these important human pathogens (Longworth and Laimins, 2004).

Papillomavirus is highly persistent in the environment, on contaminated objects, linen, floors. Skin infections can occur through indirect or direct contact. Transmission of human papillomaviruses is facilitated by the presence of abraded or macerated epithelial surfaces. Anogenital infections are mainly transmitted by sexual contact. HPV DNA is rarely detected in sexually inexperienced young women. There exists a correlation between the number of sexual partners and the prevalence of HPV infection reviewed in zur Hausen (1996).

Of the more than 100 types of HPV, over 30 types can be passed from one person to another through sexual contact. Most HPV infections occur without any symptoms and go away without any treatment over the course of a few years. However, HPV infection sometimes persists for many years, with or without causing cell abnormalities.
2.6 Immune Response to HPV:

HPV infections are largely shielded from the host immune response because they are restricted to the epithelium (Stanley, 2006). Humoral and cellular immune responses have been documented, but correlates of immunity have not been established. Serum antibodies against many different viral products have been demonstrated. The best characterized and most type-specific antibodies are those directed against conformational epitopes of the L1 capsid protein assembled as virus-like particles (VLPs). Not all infected persons have antibodies; in one study, 54%-69% of women with incident HPV 16 or 18 infections had antibodies (Carter, Koutsky and Hughes, 2000). Among newly infected women, the median time to seroconversion is approximately 8 months (Ho, Studentsov and Bierman, 2004).

The nature of the interaction between papillomaviruses (PV) and their infected host has led to the identification of ways in which the viral oncoproteins can transform the infected host cells into cancer cells. As viral persistence is required for malignancy, and persistence requires avoidance of immune attack by the host, defining the relationship between PV and the immune system is also paramount in understanding tumorigenesis.

It has emerged that PV have evolved several ways in which to prevent clearance by the host immune system. The limitation of the PV replication cycle to the epithelium, together with low level expression of the virus proteins and absence of inflammation, minimizes the exposure of virus to immune cells. In addition, more recently it has been shown that, like many other viruses, PV can directly subvert the immune response, including interference with the interferon pathway, modulation
of antigen presentation, inhibition of interleukin-18 activity and down-regulation of major histocompatibility class I on infected cells. Collectively these mechanisms explain how PV lesions are able to persist for long periods of time in immunocompetent host (Obrien and Campo, 2002).

2.7 Diagnosis and screening:

The most widely used screening approach to detect squamous intraepithelial lesions is conventional cervical cytology, followed by investigation of positive women with colposcopy and directed biopsy. Although cytology-based screening programs in Europe and North America have been followed by a substantial reduction in mortality from cervical cancer (Anhang, Annekathryn, Sue and Goldie, 2004), a wide range of rates has been reported for the sensitivity and specificity of cytology in different settings. Saslow, Runowicz, Solomon, Moscicki, Smith, Eyre and Cohen (2002) revealed that the relative insensitivity of conventional cytology triggers the need of other screening tools for optimal early detection of cervical cancer.

The knowledge gained during the last 20 years on necessity of persistent high-risk HPV infection to progress to cervical cancer has provided the basis for evaluation of clinical utility of testing for cervical cancer associated HPV types (Nobbenhuis, Walboomers, Hemerhorst and Rozendaal, 1999).
2.8 HPV DNA detection assay:

Members of the HPV family do not lend themselves to culture *invitro*: hence, detection of HPV relies strictly on molecular analysis of HPV DNA sequence (Allied for cervical cancer prevention, 2004).

The US Food and Drug Administration (FDA) has approved an HPV test and allowed it to be used in conjunction with the pap smear test to screen for HPV infection in women age 30 years (Masumoto, Fujii, Mitsuya, Ishikawa, Makio Mukai, Akiko Ono, Takashi Iwata, Kaneyuki, and Shiro Nozawa, 2004).

Most exposure measurement for HPV is based on detection of the viral DNA from biopsy tissues or exfoliated cells collected from the presumptive site of infection. Positive DNA tests are markers of prevalent infection. The absence of viral DNA is usually interpreted as absence of HPV infection, although undetectable latent infection cannot be ruled out.

HPV DNA can be detected in the blood of women with more advanced cervical carcinomas but not in the blood of women with precursor cervical lesions. So the role of HPV DNA in the circulatory system appears not be of diagnostic significance and HPV DNA is only detectable in women with more advanced cervical cancers (Kay, Allan and Denny, 2005).

Several molecular techniques have been used to detect HPV in cervical samples, including Hybrid Capture II (HCII) assay and the Polymerase Chain Reaction (PCR). The most common primers used in PCR to detect genital HPV are PGMY09/11 and GP5+/6+ (De Roda Husman, Walboomers, Van Den Brule (1995) and Strauss, Jordens, Desselberger (2000). Fuessel Haws, He and Rady (2004) found that
PGMY/GP+ nested PCR detected more HPV types than did MY/GP+ nested PCR. The determination of the HPV types may be done by several methods including Type-Specific PCR (TS-PCR) and Restriction Fragment Length Polymorphisms (RFLP). The latter relies on distinct patterns of amplicon digestion with seven restriction enzymes. However, these patterns may be difficult to discriminate, especially when clinical specimens contain two or more HPV types.

Alternatively, PCR with type-specific primers targeting E6 and/or E7 non-conserved regions is a highly sensitive method that is easy to interpret and allows characterization of infections by multiple types (Lungu1, Wright and Silverstein, 1992 Kado, Kawamata and Shino, 2001).

2.9 Treatment of cervical cancer

The best results of treating of cervical cancer are obtained in oncological units staffed by pelvic surgeons and radiological oncologists. Micro-invasive (stage 1a) cancer is treated by simple total hysterectomy. Stage 1b cancer may be treated either by radical hysterectomy or by radiotherapy. Both give similar 5-years survival rates. More advanced cervical carcinoma is treated by radiotherapy, although in some centres chemotherapy is being tried.

2.10 Prevention and vaccination:

The discovery of HPV as a cause necessary of cervical cancer has opened new preventive strategies for the control of cervical cancer both in primary prevention (via the introduction of prophylactic HPV vaccines) and on secondary prevention (via the introduction of screening for HPV). The rationale for the introduction of HPV vaccination and
HPV typing in the control of cervical cancer is based on the following facts: -Genital HPV infection is one of the most common sexually transmitted diseases.

Although most HPV infections are transient, persistent infections with a subset of 15 HPV high-risk types are now considered as a necessary cause of cervical cancer (Walboomers et al 1999) and also are known to contribute to the development of other genital cancers and cancer of the oropharynx as well as being the cause of genital warts. HPV 16 and 18 are the most common types, responsible for about 70% of cervical cancers and HPVs 6 and 11 are the main cause of genital warts. The current preventive strategies based on cytologic screening have been shown to have limitations, as they have been found to be insensitive for the detection of cancer and precancer, and requiring many rounds of screening to achieve programmatic effectiveness. Three types of HPV vaccines are under development: Prophylactic vaccines aimed to prevent HPV infections and associated diseases. Therapeutic vaccines aimed to induce regression of HPV induced lesions and Chimeric (combined prophylactic and therapeutic) vaccines with both properties. Candidate prophylactic vaccines currently under evaluation are based on papillomavirus L1 virus-like particles (VLPs).

One of the vaccines currently under evaluation is quadrivalent and contains L1 VLPs of HPV 6, 11, 16 and 18. The other vaccine is bivalent and contains L1 VLPs of HPV 16 and 18. Results of ongoing trials in human volunteers have shown that these two vaccines are well tolerated, induce high serum antibody titers and show over 95% efficacy in preventing infection and their associated precancerous lesions (Villa, Costa, Petta, Andrade, Ault, Giuliano, Wheeler, Koutsky, Malm, Lehtinen, Skjeldestad, Olsson, and Steinwall, 2005 and Harper et, Franco,
Researchers at the National Cancer Institute (NCI) and elsewhere are studying how HPVs cause precancerous changes in normal cells and how these changes can be prevented. For example, HPV E6 interferes with the human protein p53. This protein is present in all people and acts to keep tumors from growing (Howley, Ganem and Kieff, 2004). This research is being used to develop ways to interrupt the process by which HPV infection can lead to the growth of abnormal cells.

In June 2006, a new vaccine produced by Merck called Gardasil that protects against certain strains of HPV was approved by the U.S. Food and Drug Administration (FDA). Gardasil prevents infection of four strains of HPV—two strains (16, 18) that cause 70% of cervical cancer cases and two strains (6, 11) that cause 90% of genital warts cases. It does not protect against all types of cervical cancer-causing HPV. Therefore, regular Pap tests remain a critical tool for early detection of precancerous cells (Garland, 2006). Gardasil should be administered in three doses over six months. Presently, there is only enough research to show vaccine effectiveness for 5 years. Further research will determine whether booster shots are needed.

Also, scientists are developing HPV vaccines that will be stable at room temperature. The goal is to develop a vaccine that does not require refrigeration for storage and distribution, which could allow for its use in many climates and locations.

Researchers at the NCI and elsewhere are also studying what people know and understand about HPV and cervical cancer, the best way to communicate to the public about the latest research results, and how
doctors are talking with their patients about HPV. This research will help to ensure that the public receives accurate information about HPV that is easily understood, and will facilitate access to appropriate tests for those who need them.
CHAPTER THREE
MATERIAL AND METHODS

1. Study area:

This study was conducted at the Department of Molecular Biology, Institute of Nuclear Medicine and Oncology (INMO), University of Gezira. Sections of paraffin embedded tissue of cervical cancer histopathologically confirmed were selected from Department of Histopathology, medical laboratory of University of Gezira, during the period from January 2006 to June 2007.

2. Study design:

It is a retrospective cross sectional laboratory-based study on histopathologically confirmed cervical cancer.

3. Selection criteria:

- Undamaged/undistorted tissue blocks (visual evaluation)
- Histology result showing the presence of cervical cancer.

4. Study population:

All study subjects were women whose age ranged between 30 to 70 years. According to the age of the study subjects, the population was divided into two groups ≥ 45 and ≤ 44 years.

Figure 1 shows the distribution of subjects who participated in this study: 30% were females their age was ≥ 44 year (12 individuals) while 70% were females whose age was ≤ 45 old (28 individuals).
A number of 21 women (52.5%) were from rural areas (villages in the Gezira state) and 19 women (47.5%) were from urban areas (towns in the Gezira state) (Table.1).

5. Degree of differentiated cells:

According to degree of cancer, study samples were divided into three types, well differentiated cells, moderately differentiated cells and poorly differentiated cells. Table 2 shows that Cells from 21 women (52.5%) were well, from 9 women (22.5%) were moderately and from 10 women (25%) were poorly differentiated cells.
Figure 1: Age distribution of patients with cervical cancer

- ≤ 44 years
- ≥ 45 years

≤ 44; 30%
≥ 45; 70%
Table 1: Distribution of the study subjects according to the location.

<table>
<thead>
<tr>
<th></th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rural area</td>
<td>21</td>
<td>52.5%</td>
</tr>
<tr>
<td>Urban area</td>
<td>19</td>
<td>47.5%</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 2: Distribution of samples according to degree of cancer.

<table>
<thead>
<tr>
<th></th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>well differentiated cells</td>
<td>21</td>
<td>52.5%</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>9</td>
<td>22.5%</td>
</tr>
<tr>
<td>poorly differentiated</td>
<td>10</td>
<td>25%</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>100%</td>
</tr>
</tbody>
</table>
6. Sample size

All cervical samples (n= 40) included in this study (embedded in paraffin wax) were previously diagnosed as squamous cell carcinoma during 1/1/2006 to 30/6/2007.

7. Study variables:

7.1 Dependent variables

- Presence of HPV
- Presence of different HPV genotypes (16/18)

7.2 Independent variables

- Age
- Geographical location
- Degree of cell differentiation

8. Methods of data collection

Demographic and clinical data were collected according to a certain questionnaire (including sample data, personal data and types of differentiated cells) and were obtained from records of the Histopathology Department of the Medical Laboratory University of Gezira.

9. Laboratory methods

9.1. Tissue sections preparation for polymerase chain reaction (PCR):

From each cervical cancer patient's paraffin block, small sections of 30-50 microns were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut with new gloves and new disposable microtome blade.
9.2 De-paraffinization and re-hydration of sections:

One ml of xylene was added two times to a screw capped Eppendorf tube containing sections of cervical cancer embedded in paraffin. Then sections were incubated at 37°C for 30 min, vortexed and centrifuged at 800g per 5 min. The pellet was re-hydrated with serial dilutions of absolute ethanol, 75% ethanol, 50% ethanol, 25% ethanol and sterile water, vortexed and centrifuged for 3 min at maximum speed after each washing and the supernatant was discarded.

9.3 DNA Extraction:

DNA was extracted according to the steps described in DNA extraction kit purchased from Sacace biotechnologies-Casera –Italy. The pellet obtained from previous steps was treated with 1500 µl of Reagent 2 (lysis buffer ), vortexed, incubated at -20°C for 5 min and centrifuged at 3000g for 15 min. The supernatant was discarded. 150 µl of Reagent 3, 25 µl of Reagent 4 (proteinase K) and 10 µl of Reagent 5 were added to the pellet and incubated at 50°C overnight. 90 µl of Reagent 6 were added to each tube, vortexed vigorously and centrifuged at 800g for 15 min. The supernatant was transferred to a new sterile 1.5 ml Eppendorf tube. 750 µl of absolute ethanol were added to each tube and mixed by inverting. Then centrifuged at 7000g for 5min and the supernatant was discarded. This step was repeated by using 1ml 70%cold ethanol and tubes were incubated with open caps for 10 min. The pellet (containing the DNA) was re-suspended in 50 µl of Reagent 7 (DNA eluent ), shaked for 2 hours using a shaker and stored at -20°C until used.
9.5 Polymerase chain reaction (PCR):

9.5.1. Amplification of HPV:

Type specific primers (primer for HPV 16 and HPV18) were used to detect HPV16 and 18 DNA in cervical cancer. Amplification was performed according to HPV16/18 kit from Sacace technologies- Casera–Italy. The final reaction volume of 40 µl containing 20 µl mix-1 (contained in PCR tubes), 10 µl of mix-2 and 10 µl of extracted DNA (sample). Negative control, positive HPV16 DNA and positive control 18 DNA tubes contained 10 µl of DNA buffer, 10 µl of HPV 16 DNA and 10 µl of HPV18 DNA respectively. Samples and controls were amplified using Gene Amp PCR system 9700. The PCR programme was described in Table.3.

<table>
<thead>
<tr>
<th>steps</th>
<th>Temperature</th>
<th>time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95°C</td>
<td>Pause</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95°C</td>
<td>5min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95°C</td>
<td>15sec</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>65°C</td>
<td>25sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>25sec</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>1min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>4°C</td>
<td>storage</td>
<td></td>
</tr>
</tbody>
</table>
9.5.2. Gel-electrophoresis:

The PCR products were visualized in 2% agarose gel with 0.5 µg/ml Ethidium bromide. Ten micro liters of 100bp DNA ladder and PCR product was loaded on the gel. Gel-electrophoresis was performed at 120V and 36mA for about 60 minutes, Pictures were taken using Gel documentation system (Gel mega, digital camera and software in a computer).

9.5.3. Interpretation of PCR results:

According to manufacture HPV16/18 kit (from Sacace technologies- Casera –Italy) manual, the PCR product length for HPV16 should be 325bp and 425bp for HPV18.
CHAPTER FOUR

RESULTS

Data presented in this study were obtained from questionnaire and laboratory investigation. All study subjects were women cases. Histopathological diagnosis was confirmed to be as squamous cell carcinoma.

1. Results according to Type Specific Polymerase Chain Reaction (TS PCR):

- Using PCR human papilloma viruses (HPV 16 and 18) were detected in 14 (35%) of the study specimens while they were not detected in 26 specimens (65%) (Figure 1).
- The PCR product size was 325 bp and 425 bp for HPV16 and HPV18, respectively. Figure 2 shows picture of PCR result.
- The HP virus was detected in 10 individuals with ages ≥45 and in 4 individuals with ages ≤44 (Table 1).
- The HP virus was detected in 8 individuals from rural areas and in 6 individuals from urban areas (Table 4).
- HPV16 and HPV18 were detected as a single infection in 8 samples (57.1%) and 4 samples (28.6%) respectively. HPV16+18 as mixed infections was detected in 2 (14.3) of positive samples (Figure 3 and Table 2).
Figure 1: The positivity of HPV 16 and 18 among patients with cervical cancer.
Figure 2: Ethidium bromide stained agarose gel 2% electrophoresis of HPV PCR products, carried out on DNA samples extracted from paraffin embedded tissue blocks, Lane M: 100bp ladder, (Arrows shows 300 and 400 band), Lane N negative control, Lane C1 positive control for HPV16, Lane C2 positive control for HPV18, Lane 1-2-3-4-5-6-7-8 extracted DNA samples.
Figure 3: The frequency of HPV16 and 18 (as a single and mixed infection) in cervical cancer patients in the positive samples to PCR test.
Figure 4: The relation between HPV types and the degree of cell differentiation.
2. Degrees of differentiated cells and types of the virus:

   In this study within the 8 samples which contained HPV16 DNA: one was moderately differentiated cells and 7 were well differentiated cells. Out of 4 samples tested positive for HPV18 DNA 2 were from moderately differentiated cells and 2 were from well differentiated cells. The 2 samples which tested positive for HPV 16 and 18 DNA were from well differentiated cells. Non of the samples that contained HPV were from poorly differentiated cells (Figure 4).

3. The relation between the location of the study subject and the PCR results:

   Table 4 shows that 8 out of 14 (total of positive samples) of study subjects were from rural areas and 6 out of 14 were from urban areas.
### Table 1: The results of HPV type specific (16 and 18) PCR and the age of cervical cancer patients

<table>
<thead>
<tr>
<th>Result</th>
<th>Age</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 44</td>
<td>≥ 45</td>
</tr>
<tr>
<td>PCR + ve</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>PCR - ve</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>12 (30%)</td>
<td>28 (70%)</td>
</tr>
</tbody>
</table>
Table 2: The relation between type of HPV and PCR used to detect HPV 16 and 18 in cervical cancer patients

<table>
<thead>
<tr>
<th>Result</th>
<th>HPV 16</th>
<th>HPV 18</th>
<th>HPV 16+18</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR +ve</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>PCR - ve</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 3: The relation between the type of HPV and the degree of differentiated cells in cervical cancer patients

<table>
<thead>
<tr>
<th>Type of virus</th>
<th>Poor</th>
<th>Mod</th>
<th>Well</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>-</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>16+18</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>-ve</td>
<td>10</td>
<td>6</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>9</td>
<td>21</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 4: HPV positivity among patients with cervical cancer from rural and urban areas

<table>
<thead>
<tr>
<th>Location</th>
<th>Result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Rural area</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Urban area</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

DISCUSSION

The number of women contracting cervical cancer every year was estimated to be 500,000 patients. The association between HPV and cervical cancer has now been assessed in various case-control and cohort studies.

High risk HPV 16 and 18 were proved to be the etiological agents for cervical cancer by many scientists (Gissmann and Gross 1985, zur Hausen, 1996 and Clifford et al 2003). This study aimed to detect and identify HPV 16 and 18 that may be present in sections of paraffin embedded tissue of cervical cancer.

The overall positivity for HPV in this research was 35% (14/40), however, the positivity for HPV that was reported before by many authors was 87% (Clifford et al., 2003), 97% (Stanczuk et al., 2003) and 94% (Ebatia, 2005). The low positivity in this study may be due to the fact that different PCR primers and diverse molecular techniques were used and their sensitivity and specificity are different. Some of the authors used HPV general primers and applied low annealing temperature to increase the sensitivity but decrease the specificity. This may lead to false positive results due to non specific amplification. Some of those authors combined two techniques PCR and Hybridization to enhance their sensitivity and specificity. The storage condition of PCR reagents may decrease their efficiency and sensitivity. Also this low rate of cervical cancer in the Sudan is possibly due to the conservative society in the Sudan that mean there are relatively no multiple sexual partners which is an important factor that places an individual at increased risk as it was
reviewed by Adam et al (2000). Additionally, cervical cancer in the Sudan may be caused by other variant of HPV 16 and 18 and could not be detected by the used primers.

The results of the present study concerned with HPV 16 and 18 frequencies, HPV 16 was identified in 57% of the positives was comparable or in agreement with those reported before. Clifford et al (2003) detected HPV16 in 46-63%, Zur Housen,1987 in 50% and Stanczuk et al (2003) in 6% of the cervix squamous cell carcinomas.

In this study, HPV 18 was identified in 29% of the positives, which is more than that reported by Clifford et al (2003) (10-14%) and Zur Housen, 1987 (20%). However, HPV18 was not detected in a study in Iran (Farjadian et al., 2003). Accordingly, HPV16 is the most common HPV type associated with cervical carcinoma in Central Sudan. The predominance of HPV 16 over the other genotypes in this study fits well with previous reports from different geographical areas; prevalence of HPV 16 was 78.3 % in Poland and 76.5 % in Germany (Bosch et al., 2002).

An interesting finding in this study was the relatively higher rate of HPV infection in the early stage of cancer (well differentiated squamous cell carcinoma). This finding reveals the importance of the use of HPV detection as screening tool for early detection of cervical cancer.

HPV 16 and 18 were detected more in women aged ≥ 45 than in women aged ≤ 44, This result shows that women ≤ 44 years have low rates and this supported the previous explanation for the lower prevalence of HPV 16 and 18 as due to the conservative nature of the Sudanese society. Also this result agrees with Ho GYF et al (1998) who reported that, at least 50 % of sexually active men and women acquire genital
HPV infection at some point in their lives. By age 50, at least 80% of women will have acquired genital HPV infection. Cervical cancer is more common in older women (≥ 45 years), suggesting infection at a younger age and slow progressing to cancer.

In this study we found that no significant differences of HPV infection among rural and urban areas were observed (p ≥ 0.05). This may be due to the fact that there is no primary health care programmes targeting specific areas in the Sudan.
CONCLUSION

Cervical cancer, which continues to plague woman all over the world, remains a major focus of cancer research. And the role of HPV 16 and 18 in association with other factors has been extensively studied in relation to cervical cancer. Since the available therapeutic modalities are still not 100% effective in curing this disease, the prevention is still advocated by gynecological oncologists.

In the present study we found that there is a strong association between HPV16 and 18 infections and cervical cancer in Sudanese women. Also this result revealed that HPV16 was the prevailing HPV genotype identified in samples collected from women with cervical cancer in Central Sudan.

Also we found that women \( \geq 45 \) have high rate of HPV cervical cancer.
RECOMMENDATIONS

1. Further study with large sample size and involving all part of the Sudan is recommended to get conclusive results on the situation of HR-HPV infection.

2. We recommend the establishment of a national cervical cancer registry system. This is of paramount importance for the control and prevention of cervical cancer.

3. We recommend a study to determine the economical importance of HR-HPV infection in the Sudan and to determine the feasibility to introduce a vaccine.
REFERENCES


The Centers for Disease Control and Prevention, HPV Vaccine Questions and Answers, June 2006.


