Chapter One

1. Introduction

Cervical cancer is a major global public health problem and the third worldwide leading cause of cancer death among women (Xie, *et al.* 2012). In developing countries cervical cancer is the second leading cause of cancer death among women (Quinn, *et al.* 2012). More than 85% of cases and deaths occur in the developing world where the availability of effective screening is limited (Schmeler, 2012).

Cervical cancer is the fourth leading cause of female genital cancers and sixth cause of female cancer death in the United States (Hyacinth, *et al.* 2012). According to the International Agency for Research on Cancer (IARC), annual worldwide registered new cases were 530,000 of cervical cancer with 275,000 mortalities (Ferlay, *et al.* 2010). In recent years in developed countries, there is a tendency to reduce the incidence of cervical cancer due to implementation of screening programs (Igissinov, *et al.* 2012). Cervical cancer is an important public health care problem in Europe. The overall incidence rate of cervical cancer in Europe is 10.6 per 100,000 women (Kesic, *et al.* 2012). Cervical cancer is the first most common cancer in women in sub-Saharan Africa followed by breast cancer. In Ethiopia, the incidence of cervical cancer is high i.e. 35.9 per 100,000 women (Getahun, *et al.* 2013). South Africa has a high incidence of cervical cancer, with an age-standardized rate of approximately 27 per 100,000 (Botha and Doches, 2012) in which the cervical cancer is the most common female cancer (Tum et al. 2013). Cervical cancer in Sudan ranks as the second most common cancer among women with age-standardized incidence of 15 per 100,000 and age-standardized mortality of

25 per 100,000 (Ahmed, *et al.* 2012). The Asia Oceania region contributes to more than 50% of cervical cancer cases worldwide (Garland, *et al.* 2012). One-third of the world cervical cancer burden is endured in India, Bangladesh, Nepal and SriLanka (Sankaranarayanan, *et al.* 2008).

Cervical cancer ranks as the 3rd most frequent cancer among Yemeni women and the 10th most frequent cancer among women between 15 and 44 years of age (Castellsague, *et al.* 2007).

The most well-known cause of cervical cancer is the human papillomavirus (HPV) infection. Persistent HPV infection promotes the development of precancerous lesions to cervical cancer (Kwan, *et al.* 2013). HPV infection is the most common sexually transmitted disease worldwide and there is a strong link between certain high-risk viral types and cervical carcinogenesis (Carvalho, *et al.* 2010). Anogenital HPVs which are primarily mucosotropic are classified as high and low risk according to their relationship with benign or malignant proliferative lesions (DeVilliers, *et al.* 2004). HPVs have been associated with many proliferative lesions, with condyloma *acuminatum* being the most common, as well as with different types of cancer including cervical, vaginal, vulvar, penile, anal, oropharyngeal, buccal cavity and larynx **(**Montaldo, *et al.* 2007). Among them, uterine cervix carcinoma is particularly important due to its high incidence and its high mortality rate. In most cases, tumors evolve slowly and can be prevented by identifying precursor lesions in the cervical epithelium as early as possible, allowing for effective treatment before local invasion and spread of the disease (Nara de, *et al.* 2010). HPV 16, HPV 18, HPV 31, HPV 58 and HPV 52 are the five most common HPV types in cytologically normal women. They represent 50% of all HPV infections. However, certain HPV genotypes from

cervical cytologic samples vary between different geographical regions and show a strong correlation with cervical cancer incidence (Benjamin, *et al.* 2009). HR-HPV 16 and 18 are estimated responsible for nearly 70% of cervical cancer cases worldwide (Benjamin *et al.* 2012). HPV16/18/45 accounted for two-thirds of the HPV types found in invasive cervical cancer in Mali and Senegal (Ndiaye *et al.* 2012). Data is not yet available on the HPV burden in the general population of Yemen (Castellsague, *et al.* 2007). However, in Western Asia, the region Yemen belongs to, about 2.2% of women in the general population are estimated to harbor cervical HPV infection, and 66.7% of invasive cervical cancers are attributed to HPVs 16 or 18 (Maha Abdul-Aziz, 2012). However, there are many risk factors for cervical cancer, other than HPV infection which include, younger age of sexual exposure, increased number of sexual partners, long-term use of oral contraceptives, smoking, history of infertility, intrauterine device, high parity, trauma with pregnancy, low education, and low socioeconomic level (Ahmed, *et al.* 2011). Also Human immunodeficiency virus (HIV) positive women have a higher prevalence and incidence of cervical precancerous lesions than HIV negative women (Peter, *et al.* 2012).

A strong association between HPV and cervical cancer stimulated the development of several diagnostic tests, particularly those based on molecular biology (Carestiato, *et al.* 2006). Cervical biopsy is the most important investigation in diagnosing cervical cancer. Computed tomography (CT) or magnetic resonance imaging (MRI) is often used to define lymph nodes status and to assess the extent of local disease (Patrick, and Michel, 2007). Several methods have been used to diagnose clinical or subclinical infection with HPVs including clinical observation, cytological screening by Papanicolaou smear (Pap smear), electron microscopy, in

situ hybridization (ISH), fluorescence in situ hypridization (FISH), immunocytochemistry, but the most perspective way of HPV diagnosis is a direct detection of DNA of the human papilloma virus of high carcinogenic risk by the polymerase chain reaction. The PCR-based methods have been used successfully for the detection and typing of genital HPV genotypes in clinical specimens such as cervical swabs or scrapes, cervicovaginal lavages, frozen biopsies and formalin-fixed paraffin-embedded tissues (Kosel, *et al.* 2003; Hopman, *et al.* 2005; Giovanna, *et al.* 2012).

Immunohistochemistry has proved a very useful tool for the etiological diagnosis of HPV in tissue samples, in which Monoclonal antibodies directed to L1 capsid protein of high risk HPV (HR-HPV) were helpful in detection of HR-HPV infections in intra -epithelial neoplasia (Sapp, *et al.* 1994). Immunohistochemical and molecular studies can provide sufficient information for accurate diagnosis even on small biopsy specimens (Giovanna, *et al.* 2012). Screening of cervical cancer is a public health initiative based on the assumption that prevention is better than cure and that early diagnosis allow for treatment while primary pathologic process is still reversible. Screening test is ideally be inexpensive, specific, sensitive, and free risk, they should also enjoy a high level of acceptability to the population (Muchiri, *et al.* 2006).Traditionally, Pap smear, combined with treatment of cervical precancerous and early stage cancer, has been successful in preventing up to 80% of invasive cervical cancer cases in developed countries. In developing countries, however, high rates of cervical cancer mortality persist due to lack of effective screening programs and low uptake of Pap smear testing (Anne, *et al.* 2012). Preparing women with strategies to complete a mammogram and Pap test is an important approach to

enhancing self-efficacy and increasing screening behaviors (Kessler, 2012). Cervical cancer prevention is also being revolutionized by the availability of two new technologies: HPV testing and HPV vaccination. Currently approved HPV vaccines are effective against infections caused by HPV types 16 and 18, which cause about 70% of cervical cancers worldwide (Marie, *et al.* 2011). However, an effective vaccine against the five most common HPV types (16, 18, 33, 45 and 31) could prevent approximately 90% of cervical cancer cases that occur worldwide. Conversely, regional variations in the distribution of certain types of HPV should be considered in the development of vaccines tailored to different geographic regions (Munoz, *et al.* 2003). As a result, there is a need to study the prevalence of different HPV types in different geographic areas particularly, in less studied regions such as Yemen.

Chemoradiotherapy is the standard of care for locally advanced and early stage cancers with poor prognostic factors. Chemotherapy is palliative only in patients with recurrent or metastatic disease (Patrick, and Michel, 2007). The prognosis of cervical cancer patients depends on tumor stage and tumor size, but nodal status remains the single most important prognostic factor. Nodal tumor involvement occurs in up to 27% of early stage cervical cancer patients and in these patients, radiotherapy or chemoradiation is the primary treatment of choice (Joost, *et al.* 2011).

Many studies have been performed to connect the infection with HPV to the etiology of cervical cancer and to determine the prevalence of HPV genotypes in different parts of the world, but there are no such studies in Yemen. However, there is a lack of data relating cervical cancer to specific causative agent as well as, to the role of HR-HPV sub-types. Consequently, studies exposing the etiology of cervical

cancer are very important, therefore, the objective of this study was to determine the most frequent cervical cancer related HPV genotypes in Yemen.

1.2. Rationale

Cervical cancer is one of the most serious problems worldwide, and there is a strong association between HPV and cervical cancer which gives a reason to be a target for researchers to find out new ways for early diagnosis, prevention, treatment and follow up. It is now known that 99% of cases of cervical carcinoma are caused by infection with twelve high risk genotypes of the HPV. Identification of these high-risk genotypes is very valuable in the management of cervical carcinoma, both as a prognostic indicator and as a secondary screening test. However, regional variations in the distribution of certain types of HPV should be considered in the development of vaccines tailored to different geographic regions. Many studies have been conducted to determine the most frequent HPV genotypes in different parts of the world, but there are no such studies in Yemen. Therefore this was the first study to determine the most frequent HPV genotypes in Yemen in order to develop future vaccination strategies in this country.

1.3. Objectives

1.3.1. General objective

This study aimed to determine the molecular genotyping and immunophenotyping of high risk HPV types among Yemeni women diagnosed with cervical cancer

1.3.2. Specific objectives

1- To detect and identify HPV genotypes using molecular (PCR) and immunohistochemical methods.

2- To compare between immunohistochemical and molecular methods.

3- To correlate the types of HR-HPV with types of cervical cancer.

4- To correlate the types of HR-HPV and types of cervical cancer with age and geographical regions of Yemen.

Chapter two

2. Review of literature

2.1. Basic structure and histology of Female genital tract:

The female reproductive system is composed of the ovaries, uterine tube, (oviducts), uterus, vagina, and external genitalia (Paulsen, 2010). The uterus is a pear-shaped muscular organ located in the pelvis, anterior to the rectum and posterior to the bladder (Martini and Nath, 2009; Marieb and Hoehn, 2010; Paulsen, 2010). The uterus receives the right and left uterine (fallopian) tubes and it is lined by columnar epithelium and at its lower end it open into the vagina (Stevens and Lowe, 2005). The uterus can be divided into three parts: the funds, the body and the cervix. Whereas, the funds and body have the same histological structure, that of the cervix is different (Stevens and Lowe, 2005; Martini and Nath, 2009).The exposed area of the female genital organs are lined by stratified squamous epithelium which is keratinizing over the labia and non-keratinizing over the vestibule (Bancroft and Gamble, 2002). The vagina is lined with thick stratified squamous epithelium that contains abundant glycogen and usually kept moist by secretion from the uterine and cervical glands (Koss, 1992). The actual endometrium epithelial surface is made of columnar cells and is highly responsive to hormonal changes, some are ciliated (not prominent). Thus endometrium consists of glands and stroma. The stroma is composed of many small fibroplastic cells along with scattered lymphocyts, macrophages and blood vessels (Monga, 2006).

The fallopian tube is lined by columnar epithelium, some with cilia and some with secretary function (Bancroft and Gamble, 2002).

2.2. The cervix: anatomy, histology and physiology:

The cervix is the inferior or lower part of the uterus extending into the vagina as a short cylinder with a narrow, slit-like lumen. The cavity of the cervix called the cervical canal, communicates with vagina via the external os and with the cavity of the uterine body via the internal os (Steven and Lowe, 2005; Martini and Nath, 2009; Kerr 2010; Marieb and Hoehn, 2010). The mucosa of the cervical canal contains cervical glands that secrete mucus that fills the cervical canal and covers the external os, presumably to block the spread of bacteria from the vagina into the uterus (Marieb and Hoehn, 2010). The cervix is cylindrical and symmetrical being about 3 cm long and 2-2.5 cm in diameter, but become more barrel-shaped after pregnancy and parturition (Steven and Lowe, 2005). The external surface of the part of the cervix that protrudes into the vagina is the ectocervix and the lining of the lumen is the endocevix, in which the ectocervix is covered by non-keratinizing stratified squamous epithelium continuous with that of the vagina at the vaginal fornices, while the endocervical canal that runs between the uterine and the vaginal cavities is lined by single layer of tall columnar mucus-secreting epithelium, the columnar epithelium of the endocervical canal and the squamous epithelium of the ectocervix meet at the squamocolumnar junction of the cervix that is an important and common site of dysplasia that may become malignant (Stevens and Lowe, 2005; Kerr, 2010).

2.3. Female genital tract disorders:

2.3.1. Infection of vagina:

2.3.1.1. Bacterial vaginosis (B.V): (Gardnerella Vaginitis)

Is a very common cause of non-specific vaginitis, commonly associated with a thin milky white vaginal discharge (Stevens, *et al*. 2009), this occurs due to a loss of

normal vaginal lactobacilli, and consequent overgrowth of anaerobes (eg: prevotellabivia, peptostreptococcus spp.) (Schneider, *et al.* 2009). This condition is most often not associated with clinical signs of inflammation (such as vaginal wall erythema and leukocytosis), thus the term vaginosis is used instead of “Vaginitis” (Mashburn, 2006).

2.3.1.2. Vaginitis:

Bacterial Vaginitis, also called aerobic vaginitis is not common condition and sometimes is confused with BV. GroupB *Streptococci, alphahaemolytic Streptococci, Echerichia coli* and *Staphelococcus aureus* may cause bacterial vaginitis (Donders, 2007).

2.3.1.3. Candidiasis (Moniliasis):

The most common vaginitis is that caused mainly by Candida albicans (89%) and *Candida glabrata* 5%, other species of candida genus such as *C. tropicalis*, C. *parapsilosis,* and *C. krusei*, are estimated to constitute less than 1% of each and *Saccharomycescerevisiae* 1-2% (Ferrer, 2000; Edwards, 2004; Sobel, 2007). *C. albicans* is a normal commensal in vagina, but its proliferation is usually suppressed by the normal vaginal flora. C. vaginitis has been associated with antibiotic therapy, pregnancy, diabetes mellitus, oral contraceptive use, oestrogen therapy, chronic stress and immunosuppresion (Sobel, 2007; Schneider, *et al*. 2009; Stevens, *et al*. 2009). Candidiasis is characterized by white, patch-like mucosal lesions, a thick white discharge, and vulvoginal pruritus (Schneider, *et al*. 2009).

2.3.1.4. Trichomoniasis:

Is the second most common type of vaginitis that is caused by *Trichomonas vaginalis* which is a sexually transmitted protozoon infection that can be

asymptomatic in 50-75% of the cases (Edwards, 2004; Donders, 2007; Schneider *et al.* 2009). The vaginal mucosa becomes red and inflamed, with frothy white discharge on its surface (Stevens, *et al*. 2009).

2.3.1.5. Herpes simplex virus (HSV) infection:

HSV2 infection accounts for the majority of genital herpes cases and is spread by sexual contact and produces erosive vaginal lesions, small vesicles and shallow ulcers that can involve the cervix, vagina, clitoris vulva, urethra and perianal skin; multinucleated giant cells with viral inclusions are found in cytological smears from lesions (Schneider, *et al*. 2009; Stevens, *et al*. 2009).

2.3.1.6. Toxic shock syndrome:

This condition was associated with the use of highly absorbent tampons, and it is caused by exotoxin produced by staphylococcus aureus, which grow in the tampon (Schneider, *et al*. 2009).

Less common cases of vaginal infection include the gonococcus (usually secondary to gonococcal cervicitis), mycoplasma and HPV (usually associated with extensive vulval and perineal condylomata which extend up to the vagina at late stage (Stevens, *et al*. 2009). Primary tumors of the vagina in adults are exceptionally rare, but the vagina is frequently the site of metastases, particularly from malignant tumors of the cervix, endometrium and ovary. The main primary vaginal tumors are squamous cell carcinoma and even more rarely, adenocarcinomas. Clear cell carcinoma of the vagina is seen in women exposed in *utero* to the synthetic estrogen *diethylstilboestrol*, preceded by replacement of normal vaginal epithelium by glandular epithelium, termed vaginal adenosis (Stevens, *et al*. 2009).

2.3.2. Cervical disorders:

2.3.2.1. Cervicitis:

Is an acute or chronic inflammation of the cervix. Acute cervicitis may result from the direct infection of the cervix or may be secondary to vaginal or uterine infection (Porth and Gaspard, 2004; Porth and Matfin, 2009). Cervicitis most often involves the endocervix and it is often asymptomatic, and may be manifested by cervical discharge (Schneider, *et al*. 2009). It may be caused by variety of infective agents, including *C. albicans, T. vaginalis, Neisseria gonorrhoeae, Gardnerllavaginalis, Chlamydia trichomatis, Ureaplasma urealyticum, HSV and HPV* (Rubin, *et al*. 2005; Schneider, *etal*. 2009; Kumar, *et al*. 2010). Some agents are sexually transmitted; others may be introduced by foreign bodies, such as residual fragments of tampons and pessaries (Rubin, *et al.* 2005). The two most common specific infections are HSV infection which causes epithelial ulceration and HPV infection which is recognized as the major etiological factor for cervical cancer (Levison, *et al*. 2008; Kumar, *et al*. 2010). These pathogens can invade external stratified squamous epithelium of the ectocervix of the cervical canal, although only *C. trachomatis and N. gonorrhoeae* can infect the endocervical single-layered columnar epithelium (Pudney, *et al*. 2005). In acute cervicitis, the cervix is grossly red, swollen, and edematous with copious pus “dripping” from the external os and microscopically the tissues exhibit an extensive infiltrate of polymorphonuclear leukocytes and stromal edema. In chronic cervicitis which is more common, the cervical mucosa is hyperemic and there may be true epithelial erosions; microscopically, the stroma is infiltrated by mononuclear cells, principally lymphocytes and plasma cell. The metaplastic squamous epithelium of the transformation zone may extend into the

endocervical glands, forming clusters of squamous epithelium with slightly enlarged nuclei, which must be differentiated from carcinoma (Rubin, *et al*. 2005).

2.3.2.2. Erosion:

Is characterized by columnar epithelium replacing squamous epithelium, grossly resulting in an erythematous area, sometimes it is a manifestation of chronic cervicitis (Schneider, *et al*. 2009).

2.3.3. Benign tumor and tumor like conditions:

2.3.3.1. Endocervical polyp:

Endocervical polyp is the most common lesions of the cervix (Porth and Gaspard, 2004; Rubin, *et al*. 2005; Porth and Matfin, 2009). It is a benign exophytic growth that occurs in 2-5% of adult women (Kumar, *et al*. 2010). Appears as a single smooth, rounded, pear-shaped or lobulated mass, typically less than 3 cm in greatest dimension (Rubin, *et al*. 2005; Stevens, *et al*. 2009); it tipically manifestes as vaginal bleeding or discharge (Rubin, *et al*. 2005; Kumar, *et al*. 2010). Most polyps develop as a result of inflammatory hyperplasia of the endocervical mucosa (Porth and Matfin 2009; Stevens, *et al*. 2009; Kumar*, et al*. 2010), in which the lining epithelium is mucinous with varying degrees of squamous metaplasia (Rubin, *et al*. 2005).

2.3.3.2. Leiomyoma:

Leiomyoma of the cervix can bleed or prolapse into the endocervical canal, an event that leads to uterine contraction and pain resembling the early phase of labor (Rubin, *et al*. 2005). Leiomyomas may occur in the cervix but are less common at this site than in the uterus. It is derived from the smooth muscle of the cervical wall and they expand the cervix asymmetrically producing distortion and compression of the endocervical canal (Stevens, *et al*. 2009).

2.3.3.3. Microglandular endocervical hyperplasia:

Is a benign condition showing closely packed glands that lack an intervening stroma and display a neutrophilic infiltrate (Rubin, *et al*. 2005). It is induced by hormonal changes in which the cervical crypts multiply and become architecturally disordered, the lesion may be asymptomatic, but there is often a complaint of an excessive mucous vaginal discharge, and in severe cases the cervix looks to be the seat of numerous small endocervical polyps (Sevens, *et al*. 2009), and it is typically associated with progestin stimulation. It usually occurs during pregnancy and the post-partum period, and in women taking oral contraceptives (Rubin, *et al*. 2005; Stevens, *et al* 2009).

2.3.4. Premalignant neoplasms:

2.3.4.1. Cervical intraepithelial neoplasia (CIN):

CIN is defined as a spectrum of intraepithelial changes that begins with minimal atypia and progress through stages of more-marked intraepithelial abnormalities to invasive squamous cell carcinoma. CIN, dysplasia, carcinoma in situ, and squamous intraepithelial lesion (SIL) are commonly used interchangeably (Rubin, *et al.* 2005). In the CIN there is a replacement of all or part of normal squamous cervical mucosa by neoplastic cells, but with an intact basement membrane (Levison, *et al*. 2008). The neoplastic cells are identified by the presence of classical morphological features of malignancy, i.e. nuclear hyperchromasia, pleomorphism, abnormal mitosis and loss of epithelial polarity (Levison, *et al*. 2008; Schneider, *et al*. 2009). The lesion is then graded on the basis of proportion, in thirds of the epithelium that is occupied by abnormal cells showing no evidence of cytoplasmic maturation (Levison, *et al*. 2008). This changes takes place in the metaplastic epithelium of

transformation zone of the cervix and is usually associated with infection by HPV (Stevens, *et al.* 2009), in which there is a major association HPV infection types 16, 18, 31, or 33 (Schneider, *et al.* 2009). Dysplasia can progress through mild, moderate, and severe forms to carcinoma in situ and is classified as CIN, with subtypes of CIN1, CIN2, or CIN3, depending on the extent of epithelial involvement. CIN3 (carcinoma in situ) is characterized by atypical changes extending through the entire thickness of the epithelium (Stevens, *et al*. 2009; Schneider, *et al*. 2009). The oldest classification system classified lesions as having mild dysplasia on one end and severe dysplasia/carcinoma in situ on the other. This was followed by CIN classification, with mild dysplasia termed CIN1, moderate dysplasia CIN2, and severe dysplasia CIN3, because the decision with regard to patient management is two-tiered, the three-tier classification system has been recently simplified to a two-tiered system, with CIN1 renamed low-grade squamous intraepithelial lesion (LSIL) and CIN2 and CIN3 combined into one category referred to as high-grade squamous intraepithelial lesion(HSIL) (Porth, and Matfin, 2009; Kumar, *et al.* 2010).

Table (2.1): Classification system for premalignant squamous cervical lesions: (Porth and Gaspard, 2004, Kumar, *et al.* 2010).

Dysplasia/Carcinma in situ	CIN	SIL	Extent of involvement
Mild dysplasia	CIN1	LSIL	Initial one third of epithelial layer
Moderate dysplasia	CIN2	HSIL	Initial two thirds of epithelial layer
Severe dysplasia	CIN3	HSIL	Full-thickness involvement
Carcinoma in situ	CIN3	HSIL	Full-thickness involvement

2.3.5. Cervical cancer:

Occurs at the cervix, which is the lower end of the opening of the vagina. It is the third most common cancer of female reproductive organs. Usually affect women of middle age or older, but it may be diagnosed in any reproductive-aged woman (Arends, *et al.* 1990).

2.3.5.1. Historical Over view:

The understanding of cervical cancer was poor until the 1960s, advances were made in approaches to treatment in the 1970s, the etiology began to be understood in the 1980s, and the molecular biology of cervical neoplasia became better defined in the 1990s. Cervical cancer is now a disease which can at least theoretically be prevented by screening the population at risk, detecting cervical cancer precursors, and by appropriately treating those precursors and preventing the development of invasive cancer (Arends, *et al*. 1990).

2.3.5.2. Histology of Cervical Cancer:

Around two thirds of cervical cancers are squamous cell carcinoma (SCC). Adenocarcinoma is the next most common histology (around 15%); a further 15% are poorly specified carcinomas (Quinn, *et al*. 2001).

2.3.5.3. Squamous cell carcinoma:

Squamous cell carcinoma is the most common histologic subtype of cervical cancer (Porth and Gaspard, 2004; Rubin, *et al.* 2005; Lopez, *et al*. 2005; Levison, *et al.* 2008; Stevens, *et al*. 2009; Kumar, *et al* 2010; Howlader*et al.* 2013), accounting for approximately 80% of cases. HSIL is an immediate precursor of cervical squamous cell carcinoma (Schneider, *et al*. 2009; Kumar, *et al*. 2010). These lesions arise from the squamocolumnar junction and on histologic examination, squamous

cell carcinomas are composed of nests and tongues of malignant squamous epithelium, either keratinizing or non-keratinizing invading the underlying cervical stroma (Goellner, 1976; Zaino, *et al.* 1992; Stevens, *et al.* 2009; Kumar, *et al.* 2010). In the center of some of the nests, the squamous cells appear to differentiate and degenerate. Keratinizing carcinoma is characterized by cells with very hyperchromatic nuclei and densely eosinophilic cytoplasm growing in irregular invasive nests. Many of these nests have central “pearls” that contain abundant keratin. The average age of patients with squamous cell carcinoma is 51.4 years (Kufe, *et al*. 2003).

2.3.5.3.1. Invasive Cervical Carcinoma:

Invasive carcinoma develops when malignant epithelial cells break through the basement membrane and spread to the cervical stroma. As the malignancy grows, it may produce a visible ulceration or an exophytic mass, or it may extensively infiltrate the endocervix, causing the cervix to expand and harden. Anterior tumor growth results in bladder involvement manifested by urinary frequency, hematuria, a vescicovaginal fistula, or obstructive uropathy. Posterior tumor growth causes rectal extension, which leads to tenesmus, rectal bleeding, or a rectovaginal fistula. Lymphatic spread of the carcinoma occurs with sequential involvement of pelvic, para-aortic, mediastinal, and supraclavicular lymph nodes. Hematogenous dissemination usually occurs late in the course of the disease and most commonly involves the lungs, bones, and liver (Arsenio, *et al.* 2005).

2.3.5.3.2. Verrucous carcinoma:

Verrucous carcinoma is an extremely well-differentiated variant of squamous-cell carcinoma. This tumor may invade the vagina or endometrium but usually does not metastasize to the lymph nodes (VanNagell, *et al*. 1988, Berek and Hacker,

2005), but local invasion can be extensive. Histologically, the epithelium lacks cytologic atypia and mitotic activity, and the epithelial papillae lack a central fibroconnective tissue core. Mitotic activity may be evident in cells at the base of the tumor, and invasive nests of epithelium are observed along with well-circumscribed nests with a clearly visible or defined tumor-stroma interface. The inflammatory reaction at the epithelial stromal junction is marked (Kufe, *et al*. 2003).

2.3.5.3.3. Papillary Squamous Cell Carcinoma:

Papillary squamous cell carcinomas of the uterine cervix with transitional or squamous differentiation often resemble transitional cell carcinomas of the urinary tract. Invasive papillary transitional cell carcinomas of the uterine cervix are potentially aggressive carcinomas. It is important to distinguish these carcinomas from benign squamous papillomas and condyloma acuminate (Randall, *et al*. 1986; Kufe, *et al*. 2003).

2.3.5.3.4. Lymphoepithelioma-Like Carcinoma:

Lym-phoepithelioma-like carcinomas are histologically similar to lymphoepitheliomas arising in the nasopharynx and salivary glands. These carcinomas are usually well circumscribed and composed of undifferentiated cells. The cells are surrounded by inflammatory infiltrates composed of lymphocytes, plasma cells, and eosinophils (Mills, *et al*. 1985; Kufe, *et al.* 2003).

2.3.5.4. Adenocarcinoma:

The second most common tumor type is cervical adenocarcinoma, which constitute about 15 to 20% of cervical cancer cases (Kumar, *et al* 2010), and develops from a precursor lesion called adenocarcinoma in situ, which arise from the endocervical columnar cells (Greer, *et al*. 1989; Lopez, *et al*. 2005; Stevens, *et al*.

2009; Kumar, *et al*. 2010). Adenocarcinomas are characterized by proliferation of glandular epithelium composed of malignant endocervical cells with large hyperchromatic nuclei and relatively mucin-deplated cytoplasm, resulting in dark appearance of the glands, as compared with the normal endocervical epithelium (Kumar, *et al.* 2010). Over the past few decades, the percentage of adenocarcinomas has increased because, compared with squamous-cell carcinomas, they are more difficult to detect at a preinvasive stage (Young and Scully, 1990; Disaia and Creasman, 2002; Rubin, *et al.* 2005; Stevens, *et al*. 2009). Compared with invasive squamous carcinoma, adenocarcinoma tends to metastasized to lymph nodes earlier and to be less radiosensitive, although most clinical studies on cervical neoplasia have involved patients with squamous-cell carcinomas, patients with adenocarcinomas are generally treated similarly (Eifel, *et al.* 1990; Stevens, *et al.* 2009). It was reported that adenocarcinoma has a worse prognosis than squamous-cell carcinoma (Gusberg, *et al*. 1988; Eifel, *et al*. 1990; Stevens, *et al*. 2009; Lee, *et al*. 2011).

Other types of epithelial carcinomas of the cervix are less common that account for the remaining 5% of cases, but have important clinical implications (Kurman, *et al.* 1992; Kumar, *et al.* 2010).

2.3.5.4.1. Mucinous Adenocarcinoma:

Mucinous adenocarcinoma is the most common type of cervical adenocarcinoma (Hurt, *et al*. 1977, Saigo, *et al*. 1986). In the WHO classification, the first type of mucinous adenocarcinoma is composed of cells that resemble the columnar cells of the normal endocervical mucosa and is referred to as the endocervical type. The second type is termed the intestinal type because it is

composed of cells similar to those present in adenocarcinomas of the large intestine. A third type is composed of signet-ring cells and designated the signet-ring type. Frequently, mucinous adenocarcinomas are a mixture of these cell types (Kufe, *et al*. 2003).

2.3.5.4.2. Adenocarcinoma in situ (AIS):

Adenocarcinoma *in situ* (AIS) of the cervix was first described by Friedell and McKay in 1953, with few subsequent investigations of this entity during the next 20 years. In the late 1970s, there was renewed interest in characterizing precursor lesions of invasive adenocarcinoma with an intent either to invoke a unifying theory of a common sub-columnar reserve cell for all types of cervical cancer or to categorize lesions in a fashion analogous to precursors of squamous carcinoma of the cervix (Richard, 2000). This is diagnosed when tall, irregular columnar cells with increased mitotic activity replace the normal endocervical glands but maintaining the normal branching pattern without stromal invasion. About 50% of women with cervical AIS also have squamous CIN and AIS is often an incidental finding in patients operated on for squamous carcinoma. AIS is multifocal in origin and grows continuity (Mehta and Bansal, 2004).

2.3.5.4.3. Adenoma Malignum:

The term adenoma malignum of the cervix was first used in 1870 by Gusserow to describe a very highly differentiated adenocarcinoma. These tumors represent approximately 1% of adenocarcinomas of the cervix (Berek and Hacker, 2004). It is difficult to distinguish cytologically between adenoma malignum and normal endocervical glands. Thus adenoma malignum is also referred to as minimal-deviation adenocarcinoma. A distinguishing feature of adenoma malignum is a bizarre

and irregular glandular branching pattern. These irregular glands invade deeply into the stroma, and diagnosis requires a large tissue specimen (Kaku, et al. 1983; Gilks, et al. 1989). Clinically, patients usually present with watery or mucous discharge or with abnormal uterine bleeding. On physical examination, the cervix is usually firm and indurated (Berek and Hacker, 2004).

2.3.5.4.4. Clear cell adenocarcinoma:

Clear cell adenocarcinoma of the cervix was rare until 1970, when the incidence rose because of its association with in *utero* exposure before the eighteenth week of pregnancy to *diethylstilbestrol* and related nonsteroidal estrogens. The tumor occurs in two distinct age groups: those younger than 24 years and those older than 45 years. The latter are unrelated to in *utero diethylstilbestrol* exposure, but even in young women, there is no history of hormone exposure in 25% of cases (Berek and Hacker, 2004).

2.3.5.5. Other epithelial tumors:

2.3.5.5.1. Adenosquamous carcinomas:

Adenosquamous carcinomas represent approximately 20% to 30% of all adenocarcinomas of the cervix (Berek and Hacker, 2005), and contain malignant squamous and glandular components in the same tumor (Glucksmann A, Cherry, 1956; Kumar, *et al.* 2010). These tumors are associated with a higher risk of pelvic lymph-node metastasis than squamous-cell carcinomas or adenocarcinomas, but this finding has not had any significant effect on survival rates (Shingleton, *et al*. 1981; Yazigi, *et al*. 1990; Hale, *et al*. 1991).

2.3.5.5.2. Glassy-cell carcinoma:-

Glassy-cell carcinoma is a poorly differentiated form of adenosquamous carcinoma that responds poorly to surgery and radiation therapy (Maier and Norris, 1982; Disaia and Creasman, 2002; Berek and Hacker, 2005). These carcinomas are made up of large uniform polygonal cells with a finely granular cytoplasm of the ground-glass type, hence the term “glassy-cells”. Similar to other undifferentiated tumors, glassy-cell carcinomas spread early and are aggressive (Seltzer, *et al.* 1979; Gallup, *et al.* 1987; Zolciak-Siwinskaand Jonska-Gmyrek, 2014).

2.3.5.5.3. Small-cell carcinomas:

Small cell cancers are rare, heterogeneous group of tumor, representing 0.5% to 5% of all invasive cervical cancer (Berek and Hacker, 2004). Small-cell carcinomas are distinctive and, collectively, have a very poor prognosis, contain small anaplastic cells with scant cytoplasm; the most aggressive tumors are those with neuroendocrine differentiation (VanNagell, *et al*. 1988; Silva, *et al*. 1989). This group should be distinguished from the poorly differentiated squamous-cell carcinomas with small cells and the adenocarcinomas with carcinoid features (Lopez, *et al*. 2005). Small-cell carcinomas tend to behave very aggressively and are frequently associated with widespread metastasis to multiple sites, including bone, liver, skin, and brain (Morris, *et al*. 1992; Abeler, *et al.* 1994).

2.3.5.5.4. Non-Small-Cell Neuroendocrine Carcinoma:

Non-small-cell neuroendocrine carcinomas of the cervix have been reported (Silva, *et al*. 1984, Gilks, *et al.* 1997). The tumors contain intermediate to large cells, high-grade nuclei, and eosinophilic cytoplasmic granules of the type seen in neuroendocrine cells. A trabecular pattern is frequently evident, with or without

glandular differentiation. Terminology for cervical neuroendocrine tumors is confusing, and many pathologists are recommending application of the classification system used for the more common pulmonary neuroendocrine tumors (ie, typical carcinoid tumor, atypical carcinoid tumor, large cell neuroendocrine carcinoma, and small [oat]-cell carcinoma) (Albores, *et al*. 1997).

2.3.5.6. Malignant non-epithelial tumors:

These cervical malignancies include sarcomas, malignant melanomas, lymphomas, mixed mllerian tumors, germ-cell tumors, and trophoblastic tumors (Lopez, et al. 2005, Berek and Hacker, 2005).

2.3.5.7. Stages of Cervical Cancer:

Cervical cancer is divided into stages:

Stage 1 is often divided into 1a and 1b and means the cancer remains within the cervix. In Stage 2 the cancer has begun to spread around the cervix into the surrounding tissues, this stage can also be divided into 2a and 2b, In Stage 3 the cancer has spread into the pelvis. By Stage 4 the cancer is advanced and has spread into other body organs (Quinn, *et al.* 2001).

Table (2.2): Staging of Carcinoma of the Cervix Uteri: International Federation of Gynecologists and Oncologists (FIGO) (Gusberg, *et al.* 1988; Shepherd, 1995; Disaia and Creasman, 2002; Berek and Hacker, 2005; Rubin, *et al*. 2005; Huether and McCance, 2008; Shahabi, *et al*. 2010; Kumar, *et al.* 2010).

Stage 0	Carcinoma in situ, intraepithelial carcinoma (cases of stage 0 tumors should not be included in any therapeutic statistics)
1a	Carcinoma is stricutly confined to the cervix (extension to the corpus should be disregarded)

1a1	Invasive cancer identified only microscopically; all gross lesions, even with superficial invasia, are stage Ib cancers; invasion is limited to measured stromal invasion with a maxium depth of 5 mm and no wider than 7 mm
1a2	Measured invasion of stroma greater than 3 mm and no greater than 5 mm in depth and no wider than 7 mm
1b	Clinical lesions confined to the cervix or preclinical lesions greater than Ia
1b1	Clinical lesions no greater than 4 cm in size
1b2	Clinical lesions greater than 4 cm in size
Stage 2	Carcinoma extends beyond the cervix but has not extended on the pelvic wall; the carcinoma involves the vagina, but not as far as the lower third
2a	No obvious parametrial involvement
2b	Obvious parametrial involvement
Stage 3	Carcinoma has extended to the pelvic wall; on rectal examination, there is no cancer-free space between the tumor and pelvic wall; the tumor involves the lower third of the vagina; all cases with a hydronephrosis or nonfunctioning kidney should be included, unless they are known to be due to other cause
3a	No extension onto the pelvic wall, but involvement of the lower third of the vagina.
3b	Extension onto the pelvic wall or hydronephrosis or nonfunctioning
Stage 4	Carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum

4a	Spread of the growth to adjacent organs
4b	Spread to distant organs

FIGO = International Federation of Gynecologists and Oncologists. The depth of invasion should not be more than 5 mm taken from the base of the epithelium, either surface or glandular, from which it originates. Vascular space involvement, either venous or lymphatic, should not alter the staging.

2.3.5.8. Epidemiology of cervical cancer:

Cervical cancer accounts for 15% of all cancers in women (Gonca and Gulay, 2013) and is the second most common cancer affecting women worldwide (Mohamed, *et al*. 2012; Abdelbaset, *et al*. 2012; Qmichou, *et al*. 2013; Gonca and Gulay, 2013) accounting for about 530,000 new cancer cases and 275,000 cancer deaths among females in 2008 (Jemal, *et al*. 2011; Ya Li, *et al*. 2012; Ahmed, *et al*. 2012; Abdelbaset, *et al*. 2012; Mohamed, *et al*. 2012) and 585,278 incident cases and 327,899 deaths attributable to cervical cancer are predicted for 2010 (Yao Jia, *et al*. 2012). More than 85% of these cases and deaths occur in developing countries (Ferlay, *et al*. 2010; Ayten Dinc, 2012; Ana Pavla, *et al.* 2013; Edward, *et al*. 2013; Qmichou, *et al*. 2013). Worldwide, the highest incidence rates of cervical cancer are in Eastern, Western, Southern Africa, Central America, and South America where there is lack of screening and early detection programs (Edward, *et al*. 2013; Sahar Elderdiri, *et al*. 2013). In developed countries, cervical cancer incidences have declined, mostly due to cervical cytology screening campaigns. Cervical cancer is on the rise in the developing world, with one-seventh of the world's cervical cancer cases in China (Abdelbaset, *et al*. 2012). Cervical intraepithelial neoplasia (CIN) is

estimated to have at least 600,000 new cases per year making (pre) neoplastic cervical disease a major public health threat and heavy burden to the society, especially in some high prevalent countries, such as India , Korea and America (Ya Li Luo,*et al*. 2012). In the U.S.A there is approximately 12,710 new cases diagnosed and 4290 deaths occurring in the year 2011 (Denslow et al. 2012). Worldwide, in 2010, cervical cancer killed about 200,000 women, of whom 46,000 were aged 15-49 years in developing countries (Forouzanfar, *et al*. 2011; Xian, *et al*. 2012; Xue Xiao, *et al.* 2013). Invasive cervical cancer remains an important global cause of death, despite the declining prevalence within the United States (Dizon, *et al.* 2014). The incidence rate of cervical cancer among African American women is 11.1 per 100,000 as compared to 7.9 per100,000 among white women, which places cervical cancer as the 7th leading type of new cancer cases among African American women (Isabel, *et al*. 2013). Hispanic women living on the United States-Mexico border experience health disparities, have a higher rate of cervical cancer incidence compared to women living in non-border areas (Thompson, *et al*. 2014). In comparison to other racial and ethnic groups in the USA, Vietnamese women experience the highest incidence rate of invasive cervical cancer (Grace, *et al*. 2012). In Brazil, cervical cancer is the third most common cancer among women (Ana, *et al*. 2013).

Cervical cancer incidence rates are high in many countries in Central and Eastern Europe and Central Asia, relative to other populations on the European and Asian continents. In Central and Eastern Europe, Romania and the Former Yugoslav Republic [FYR] of Macedonia had the highest rates in 2008 alongside Bulgaria, Lithuania and Serbia, while in Central Asia, rates are elevated in Kyrgyzstan (the highest rates across the regions), Kazakhstan and Armenia. In each of these

countries, at least one woman in 50 develops cervical cancer before the age of 75 (Bray, *et al*. 2013). In Turkey, breast cancer ranks first with an incidence of 41.6% and cervical cancer is the tenth most common cancer with an incidence of 4.4% (Ayten Dinc, 2012; Gulendam, *et al*. 2014). In South Africa, cervical cancer is the second leading cause of death among women (Francis, *et al*. 2011). According to the Cancer Association of South Africa (CANSA) (2000-2001), women have a lifetime risk of 1 in 35 of getting cancer of the cervix. Thus, cervical cancer among women in South Africa is an important Public Health concerns (Edward, *et al*. 2013). In sub-Saharan Africa, invasive cervical cancer (ICC) incidence and mortality are among the highest in the world (Denny, *et al*. 2014). In Morocco, as it is the case in the other North African countries, cervical cancer is the second most common cancer among women and its incidence is the highest in this region with an age-standardized incidence rate (ASR) of 13.5 per 100 000 women (Mohamed, *et al.* 2012; Qmichou, *et al*. 2013). Also cervical cancer is reported as the most frequent malignancy among women visiting hospitals in Ethiopia (Mihret, *et al*. 2014).

Cervical cancer on the other hand, is the leading cause of reproductive tract cancers in Kenya. It is associated with high morbidity and mortality burden among women at risk yet it is largely preventable and can be effectively treated if detected (Omondi and Mkhize, 2014). In Sudan cervical cancer ranks as the second most common cancer among women (Ahmed Ibrahim, *et al*. 2012), and there are about 923 new cases representing 4.5/100,000 (Abdelbaset, *et al*. 2012), with more than two-thirds of all women with invasive cervical cancer being diagnosed at an advanced stage (stages III and IV) (Ibrahim, *et al*. 2011).

Since the introduction of organized screening in Australia in 1991, cervical cancer incidence and mortality rates among women 20 years of age and older have fallen substantially, by about 50% to date (Nayyereh, *et al*. 2012).

Cervical cancer represents a major health issue in Korea and Japan, economically developed Asian nations (Razak, *et al*. 2013), in which in Korea, cervical cancer accounted for 9.8% of new cancer cases in 2002 (Eun Cho, *et al*. 2013). The National Cancer Registry of Cancer Incidence in Malaysia (2006) showed that cervical cancer constituted 12.9% of total female cancers, and 10.5% of deaths among women in 2002 were due to cancer of the cervix (Pryma, *et al*. 2013). China has one of the highest incidence rates of cervical cancer in the world, with 135,000 new cases being detected every year (Yao, *et al*. 2012). India accounted for a quarter of both the world's estimated cervical cancer burden of 530,000 cases and 275,000 deaths in 2008. Cervical cancer is the most frequent primary site of cancer among Indian women with the estimated age standardized cervical cancer incidence and mortality rates of 27 and 15 per 100,000 women, respectively in 2008 (Ferlay,*et al*. 2010, Jissa, *et al.* 2012). Cervical cancer's crude incidence rate in Iran is 6-8 per 100,000 (Mojahed, *et al*. 2013). In 2000, cervical cancer ranked number eight or more in China, Jordan and Saudi Arabia. From GLOBOCAN, squamous cell carcinoma (SCC) of the cervix continues to be a major problem in many areas of the Asian-Pacific (Malcolm and Kazuo, 2003).

2.3.5.9. Etiology and risk factors of cervical cancer:

It is now generally agreed that persistent infection by high-risk HPV genotypes is the major cause of cervical cancer (Nyasha, *et al*. 2014). More than 120 HPV types have already been characterized, among which 40 different types infecting the genital

tract (Suthipintawong, *et al*. 2011) which classified as high-risk type (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -66 and -68) associated with cervical carcinoma vs. the low-risk type (HPV-6, -11, -40, -42, -43, -44, -54, -61 and -72) associated with genital warts (Kawana, *et al*. 2012; Sitakan, *et al*. 2013), only the high-risk (HR-HPV) types are considered as true human carcinogens. Within this group, HPV-16 and -18 are the most prevalent viral types associated with Cervical Cancer, accounting for more than 65-70% of cases worldwide (Gerardo, *et al*. 2012), followed by HPV 31 and 45. A proportion of CIN, if not detected and treated, progress to invasive cervical carcinoma over a period of 10-20 years owing to the effect of other cofactors (Wentzensen, *et al*. 2009). Cervical cancer is a multi-etiology disease and HPV infection alone is not a sufficient cause of cervical cancer. Most HPV infections regress rapidly without causing clinically significant disease (Sankaranarayanan, *et al*. 2008; Jissa, *et al*. 2012). There are several risk factors that universally predispose women to acquiring high-risk HPVs that may cause cervical cancer. These factors include multiparity, early age of first intercourse (Xian-Tao, *et al.* 2012; Nyasha, *et al*. 2014), multiple sexual partners, poor genital hygiene, use of oral steroid contraceptives, cigarette smoking, some dietary factors and infection with other sexually transmitted pathogens such as HIV, *Chlamydia trachomatis* (Jissa, *et al.* 2012; Arseniy, *et al*. 2012; Nyasha, *et al*. 2014), and herpes simplex virus which cause chronic cervicovaginal inflammation (Nyasha, *et al*. 2014). HIV-positive women are nearly five times more likely to have high-risk HPV-infection compared to HIV-negative women (Ingrid, *et al*. 2013). Alicia and his colleges in South Africa 2014 found that the HPV prevalence was higher among HIV-positive women (52.4%) than among HIV-negative women (20.8%) overall and in all age groups (Alicia, *et al*.

2014). In sub-Saharan Africa the prevalence of single and multiple HPV infections seemed higher among HIV-positive women and HPV type distribution appeared to differ according to tumor type and HIV status. HPV16, 18, 45 and 35 were the most common HPV types in sub-Saharan African women with ICC and HPV infections were more common in HIV-positive women (Denny, *et al*. 2014). Joshi and other researchers in Maharashtra, India 2014 found that Women under 30 or over 44 years, no abortions, and women with diagnosis of HIV infection within the last 5 years were at high risk of multiple oncogenic HPV infection (Joshi, *et al*. 2014).

In addition to the above factors, there are other cofactors such as host genetic factors and immunodeficiency that are considered to be also important for development and progression of cervical cancer, race and ethnicity are also important risk factors of cervical cancer (Nyasha, *et al*. 2014). Also age, education, income, lack of knowledge about screening of cervical cancer and its prevention, personal and life style factors, attitudes, ease of access and lack of patient friendly health services (JissaVinoda, *et al.* 2013). Invasive cancer case subjects who were positive for HPV16 or 18 were diagnosed at younger ages than those who were positive for other carcinogenic HPV genotypes (Cosette, *et al*. 2009). Women with adenocarcinoma were not younger than those with squamous cell carcinoma, as well, as women infected with HPV33 were older than those infected by other HPV types (Cristina Mendes, *et al.* 2013). The socio demographic risk quantification is warranted to acquire a better picture of the determinants of cervical carcinoma in low resource settings (Jissa, *et al*. 2012). Human leukocyte antigens (HLA) play important roles in presenting foreign antigens to T lymphocytes, which mediates the host cellular immune response to HPV infection. Genetic regions encoding HLA is highly

polymorphic, resulting in various products with different efficiency in presenting antigens. Therefore, HLA polymorphisms may lead to different responses to HPV infection and further contribute to the progression to cervical cancer (Xue Xiao, *et al*. 2013).

Many studies had conducted worldwide to investigate the prevalence of HPV and genotype distribution among women with cervical abnormalities and we are going to focus on some of these studies as example like in 2013 a study was conducted to determine the prevalence of human papillomavirus and its impact on cervical dysplasia in Northern Canada in which the most common HPV type detected was HPV 16 across region, the prevalence of other high risk HPV types was different (Ying, *et al*. 2013).

Also in North-East Brazil Fernandes and other researchers, found that overall HPV prevalence was 65.2% (277/425), with 85.9% (238/277) single and 14.1% (39/277) multiple infection. The most prevalent HPV types were HPVs 16, 58, 18, 31, and 45. HPV 16 was the most prevalent genotype, independently of the health status of patients. HPV 58 was the second most prevalent type in women with normal cytology and in those who had mild or moderate dysplasia. HPV 58 presented equal prevalence to HPV 18 in patients with severe dysplasia. However, it was less prevalent than HPV 18 in women with cervical cancer (Fernandes, *et al*. 2013).

Gerardo and his colleagues carried out a study in Argentina and they found that HPV was detected in 46.7% of the samples and 21 different types were found; the most frequent being HPV-16 (19.4%), 6 and 18 (5.3%), 58 (3.5%) and 31 and 33 (3.1%). In relation to HPV-16 variants, 68.2% were European and 31.8% Asian-American (Gerardo, *et al.* 2012).

In 2013 a study was performed by many researchers in France and they found that the age-standardised prevalence rates of HPV 16 and/or 18 (with or without other high-risk types) were 47.2% in (HSILs), 20.2% in LSIL and 3.9% in normal cytology. Overall HR HPV was detected in 13.7% of normal cytology. In women below 30 years of age, 64% of HSILs were associated with HPV16 and/or 18. In their study population, HPV16 was the most commonly detected type in all cervical grades with prevalence rates ranking from 3.0% in normal cytology to 50.9% in HSILs (Isabelle, *et al*. 2013).

In Spain 2013, Perez-Castro and others found that the HR-HPV prevalence was 96.8%. The most frequent genotypes were HPV 16 (48.8-58.2%) and HPV 31 (9.3%-12.1%), considering single infections or single-multiple infections, respectively (hierarchical attribution). In squamous lesions, HPV 16 prevalence in women younger than 45 years of age increased in severe lesions (CIN3-CIS/SCC, OR 4.2), and was higher than in older women (OR 5.5) (Perez-Castro, *et al*. 2013).

In 2012, a study was conducted by Tuncer and others to determine the HPV genotype distribution among Turkish women. They found that HPV was detected in 404 (22.4%) of 1797 samples studied. HPV DNA was identified in 194 cases by using HPV-Typing test but the specific genotype was not available. The most frequent genotype was HPV 16 which was observed in 103 cases (49.0%) (Tuncer, *et al.* 2012).

Women with cervical cancer in Africa usually have the following HPV types, in descending order, 16, 18, 33, 45, 35, 31, 58, and 52. On the other hand, HPVs 16, 33, 31, 18, 52, 58, 35 and 56 are those most frequently present in women with HSIL (Benjamin, *et al*. 2009).

A few studies have been done that showed the presence of high-risk genital HPV genotypes such as 16, 18, 31, 33, 52, 58 and 70 in Zimbabwean women with cervical cancer in which the prevalence of HPV DNA in women with cervical cancer has been shown to range from 63% to 98%. The high-risk HPV 16, 18, 31, 33 and 58 were the most common genotypes in all the studies (Nyasha, *et al*. 2014).

Abdelbaset and other researchers in 2012, found that the frequency of infection with HR-HPV subtypes 16 and 18 is high among Sudanese women with cervical lesions and suggests a role of HR-HPV in the development of cervical cancer in Sudan (Abdelbaset, *et al*. 2012)

Joshi and other researchers in Maharashtra, India 2014 found that HPV16 was the most common genotype, present in 11.5%, and 58.5% of women with cervical intraepithelial neoplasia (CIN) 2 and 3. Other most common high-risk HPV types in CIN 2-3 lesions were HPV 31 (22.6%); 56 (13.2%); 18 and 68a (11.3%) and 33, 35 and 51 (9.4%); and 70 (7.5%) (Joshi, *et al*. 2014).

In 2013 a study was performed by Quek and other researchers to evaluate the prevalence of human papillomavirus (HPV) types (high risk and others including coinfections) in women with invasive cervical cancer (ICC) and high-grade precancerous lesions across 5 countries in Asia, namely, Malaysia, Vietnam, Singapore, South Korea, and the Philippines, in which women older than 21 years with a histologic diagnosis of ICC and cervical intraepithelial neoplasia [CIN 2 or 3 and adenocarcinoma in situ (AIS)] were enrolled. They found that the most common types observed among ICC cases were HPV 16 (36.8%-61.3%), HPV 18 (12.9%-35.4%), HPV 52 (5.4%-10.3%), and HPV 45 (1.5%-17.2%), whereas among CIN

2/3/AIS cases, HPV 16 (29.7%-46.6%) was the most commonly observed type followed by HPV 52 (17.0%-66.7%) and HPV 58 (8.6%-16.0%) (Quek, *et al*. 2013).

2.3.6. Human Papilloma viruses:

Human Papilloma viruses belong to the family of *Papillomaviridae*, a widespread virus family that infects almost all mammalian species and birds (Konstanze, *et al.* 2014). HPV is strictly epitheliotropic and infects epithelial cells either of the skin or mucous membranes, particularly of the anogenital tract and oropharyncx (Rachel, *et al*. 2011). Based on DNA sequence analyses, over 200 different subtypes have been identified so far, of which there are 170 which are known to infect humans (Quint*, et al*. 2012; Konstanze, *et al*. 2014). More than 30 to 40 types of HPV are typically transmitted through sexual contact and infect the anogenital region (Schiffman and Castle, 2003).

Depending on the HPV subtype, the productive infection can cause the formation of benign warts or malignant tumors, such as cervical cancer. In fact, 99.8% of all cervical cancer cases are attributed to a persistent infection with a so-called high-risk HPV type. The high-risk types HPV16 and HPV18 are alone responsible for over 70% of cervical cancer cases (Konstanze, *et al*. 2014). High-risk HPVs are important risk factors for other human cancers as well, such as head and neck (HN) and colorectal carcinomas; as roughly 30 and 80% of these cancers are positive for high-risk HPVs, respectively (Venuti*, et al*. 2004; Daling, *et al*. 2004). Moreover, it was observed that the presence of high-risk HPVs serve as a prognostic factor in early-stage cervical, HN, and colorectal cancers, and could be associated with vascular invasion, lymph node metastases, and tumor size (Ala-Eddin, *et al.* 2014). HPV has been linked with an increased risk of cardiovascular disease (Kuo and Fujise, 2011).

In addition, numerous recent studies revealed that high-risk HPVs are present in human breast cancers worldwide (Glenn, *et al*. 2012).

HPVs are the most common sexually transmitted infections worldwide, with the majority of individuals who engage in sexual activity becoming infected at some point in their lifetime (Ala-Eddin, *et al*. 2014).

Table (2.3): HPV types associated with clinical diseases (Burd EM. 2003; John, *et al*. 2011).

Disease	**HPV genotypes**
Commons warts	2,7,10,26,27,29,41,57,65,77
Plantar warts	1, 2, 4, 63
Flat warts	3,8,10,26,28,38,41, 49,75,76
Genital warts	6,11,30,42,43,45,51,54,55,70
CIN	More than 30 HPV types e.g. 16,18,31,33,35,39,42,43,44,45,51,52,56.
Penile intraepithelial neoplasia	16,18
Anal intraepithelial neoplasia	16 (rarely 6,11,18,33)
Vulvar intraepithelial neoplasia	16 (occasionally 6,11)
Cervical cancer	16,18 (strong association) 31,33,35,39,45,51,52,56,58,66,68,70 (moderate association) 6,11,42,43,44 (weak association)
Miscellaneous cutaneous lesions	6,11,16,30,33,36,38,41,48,60,72,73
Recurrent respiratory papillomatosis	6,11
Epidermodysplasiaverruciformis	2,3,5,8,10,12,14,15,17,19-25,36-38, 47,50
Focal epithelial hyperplasia (Heck's	13,32

disease)	
Oral papillomas	6, 7, 11, 16, 32
Oropharyngeal cancer	16
Verrucous cyst	60
Laryngeal papillomatosis	6,11
Conjunctival papillomas/carcinomas	6,11,16
Bowen disease	6,11

2.3.6.1. Human Papilloma virus's genome:

Human papillomaviruses are small non-enveloped viruses with a circular double stranded deoxyribonucleic acid (DNA) genome, approximately 8 kilo base pairs (kb) in size. The genome includes a non-coding region (LCR = long control region) and eight open reading frames (ORF), six located in the 'early' region and two in the 'late' region (Sheila, 2010; Konstanze, *et al*. 2014). The DNA-region has binding sites for different cellular transcription factors and regulates the expression of the viral early and late genes. The early genes E1, E2, E4, E5, E6 and E7 are non-structural viral proteins responsible for viral replication and transcription, as well as for transformation, segregation, cell cycle, cell signaling, apoptosis control, immune modulation and structural modification of the infected cell (Sheila, 2010; Quint, *et al*. 2012). Most of these proteins are expressed through out the infectious cycle perhaps with reduced expression at late stages. E4 is the first virus protein expressed late in infection and expression may be accompanied by E5, which also appears later in infection (Sheila, 2010). The approximately 55 nm diameter in size HPV capsid is composed of the two structural proteins: the major capsid protein L1 and the minor capsid protein L2. The capsid contains 360 copies of L1 molecules and a so far

unknown number of L2-copies: 12, 32 or up to 72 per capsid have been identified (Konstanze, *et al*. 2014). The capsid proteins L1 and L2 are key players in early events of infection, such as virus binding at the plasma membrane, entry into the cell, and transport of the viral DNA into the nucleus (Sapp and Spoden, 2012; Roden, 2013). The capsid proteins L1 and L2 also required for virus transmission, spread and survival in the environment (Graham, 2006).

2.3.6.2. HPV Oncogenes and their interaction:

The two primary oncoproteins of high risk HPV types are E6 and E7. The “E” designation indicates that these two proteins are expressed early in the HPV life cycle, while the "L" designation indicates late expression (Ault, 2006). After the host cell is infected viral early promoter is activated and a polycistronic primary RNA containing all six early ORFs is transcribed. This polycistronic RNA then undergoes active RNA splicing to generate multiple isoforms of mRNAs (Zheng and Baker, 2006). One of the spliced isoform RNAs, E6*I, serves as an E7 mRNA to translate E7 protein (Tang, *et al*. 2006). However, viral early transcription subjects to viral E2 regulation and high E2 levels repress the transcription. HPV genomes integrate into host genome by disruption of E2 ORF, preventing E2 repression on E6 and E7. Thus, viral genome integration into host DNA genome increases E6 and E7 expression to promote cellular proliferation and the chance of malignancy. The degree to which E6 and E7 are expressed is correlated with the type of cervical lesion that can ultimately develop (Scheurer, *et al*. 2005).

2.3.6.2.1. E6 Oncoprotein:

The E6 oncoprotein of high-risk (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, *et al*.1989). E6 proteins exert their functions by interacting with cellular proteins. High-risk HPV encoded E6 protein that forms a complex with p53 leading to functional inactivation (Werness, *et al.*1990).

2.3.6.2.2. E7 Oncoprotein:

HPV E7 protein binding and degradation of the retinoblastoma protein (pRb) are necessary for its transforming activity. High-risk HPV E7 proteins interact with pRb and induce its proteolytic degradation. The E7 protein together with E6 provide the major transforming activities of HPVs. Expression of the E7 protein in the absence of other viral gene products leads to the transformation of rodent fibroblasts. While E7 alone can immortalize human keratinocytes, the presence of E6 greatly enhances the frequency at which this can occur (Hubbert, *et al*.1999).

2.3.6.2.3. Role of oncogenes in cancer:

The E6/E7 proteins inactivate two tumor suppressor proteins, p53 (inactivated by E6) and pRb (inactivated by E7) (Chaturvedi, *et al*. 2010). The viral oncogenes E6 and E7are thought to modify the cell cycle so as to retain the differentiating host keratinocyte in a state that is favourable to the amplification of viral genome replication and consequent late gene expression. E6 in association with host E6-associated protein, which has ubiquitin ligase activity, acts to ubiquitinate p53, leading to its proteosomal degradation. E7 (in oncogenic HPVs) acts as the primary transforming protein. E7 competes for retinoblastoma protein (pRb) binding, freeing the transcription factor E2F to transactivate its targets, thus pushing the cell cycle forward. All HPV can induce transient proliferation, but only strains 16 and 18 can

immortalize cell lines *in vitro*. It has also been shown that HPV 16 and 18 cannot immortalize primary rat cells alone; there needs to be activation of the ras oncogene. In the upper layers of the host epithelium, the late genes L1 and L2 are transcribed/translated and serve as structural proteins that encapsidate the amplified viral genomes. Once the genome is encapsidated, the capsid appears to undergo a redox-dependent assembly/maturation event, which is tied to a natural redox gradient that spans both suprabasal and cornified epithelial tissue layers. This assembly/maturation event stabilizes virions, and increases their specific infectivity (Conway, *et al*. 2009). Virions can then be sloughed off in the dead squames of the host epithelium and the viral lifecycle continues (Bryan, *et al*. 2001). A study in 2010 has found that E6 and E7 are involved in beta-catenin nuclear accumulation and activation of Wnt signaling in HPV-induced cancers (Rampias, *et al*. 2010).

Table (2.4): Major roles of proteins expressed by high-risk human papillomaviruses (Sheila, 2010).

protein	**Role in the virus lifecycle**
E1	Genome replication: ATP-dependent DNA helicase
E2	Genome replication, transcription, encapsidation. Regulation of cellular gene expression; cell cycle and apoptosis regulation
E4	Remodels cytokeratin network; cell cycle arrest; virion assembly
E5	Control of cell growth and differentiation; immune modulation
E6	Inhibits apoptosis and differentiation; regulates cell shape, polarity, mobility and signaling
E7	Cell cycle control; controls centrosome duplication
L1	Major capsid protein
L2	Minor capsid protein; recruits L1; virus assembly

2.3.6.4. The Human Papilloma virus Life Cycle:

HPVs are nonenveloped viruses that replicate in the nuclei of the infected host cell (Longworth and Laimins, 2004b). The productive life cycle of HPVs is directly linked to differentiation of the infected epithelial cell. Infection by papillomaviruses is believed to occur through microtraumas in the epithelium, which exposes cells in the basal layer to entry by viruses. The receptor that mediates viral entry remains unknown although heparin sulfate is likely responsible for the initial attachment of virions to cells (Howley, 1996). Once the virus has entered into keratinocytes in the basal layer, HPV genomes are established in the nucleus as replicating extrachromosomal episomes and a low level of HPV expression occurs. This viral gene expression allows for the stable maintenance of viral episomes at approximately 20 to 100 copies per cell. As these infected basal cells divide, viral genomes are replicated along with host chromosomes and distributed equally to daughter cells. One of the infected daughter cells detaches from the basal layer, migrates toward the suprabasal regions, and undergoes differentiation. The other daughter cell remains in the basal layer to provide for further cell division (Fehrmann and Laimins, 2003; Longworth and Laimins, 2004b). Normally, uninfected keratinocytes exit the cell cycle as they detach from the basement membrane and in many keratinocytes this results in degradation of nuclei in suprabasal cells. However, HPV-positive cells remain active in the cell cycle after leaving the basal layer through the action of viral proteins. Upon differentiation, suprabasal HPV-positive cells are induced to reenter S phase and activate expression of cellular factors necessary for replication to induce viral DNA amplification (Flores, *et al*. 2000). Concurrent with DNA amplification, late viral proteins including capsid proteins are expressed resulting in the assembly of

infectious virions. Following assembly, mature viruses are released from the upper layers of the epithelium through shedding (Howley 1996; Longworth and Laimins, 2004b).

2.3.7. Molecular Genetics Of cervical Cancer:

The papillomavirus is unable to reproduce itself but must reside in cellular nuclei, where the proteins encoded by its genome redirect certain cellular mechanisms to copy the viral DNA and coat it with a protein to produce the complete virion, which is the infectious agent. Two of the HPV open reading frames (ORFs) (genes) that are involved in the virus's ability to remain in the cell and duplicate itself may, under certain circumstances, interact with host-encoded proteins critical to regulating cell division (Keating, *et al*. 2001). These ORFs, known as E6 and E7, are regulated by other viral proteins known as El and E2, and among other things, form a complex with retinoblastoma protein (pRb) an important cell regulatory protein and with p53 another cell regulatory protein commonly referred to as a "molecular policeman." p53 is an extremely important call regulatory protein that is responsible for orchestrating the process through which the DNA base pairs are surveyed after DNA replication but prior to cytokinesis. Under the direction of p53 and other proteins, cells in which coding errors or other genetic defects cannot be repaired are shunted off to program cell death (apoptosis) so that mutations will not be carried through successive cell divisions. P53 is like a DNA spell check. It is now known that if the E6- and E7-encoded proteins inactivate a sufficient proportion of the cells' pRb and p53, these proteins are rendered nonfunctional, the DNA spell check no longer occurs, and genetic defects accumulate during successive cell cycles. When a sufficient number of errors in the signaling cascade involved in the control of cell division occur, the cell

becomes neoplastic (Giarre, *et al.* 2001). Now that more is known about the molecular biology of HPV and its proteins' interaction with host regulatory proteins, it is possible to think about designing genetic messages that could function as therapeutic agents by interfering with the continuing production of E6 and E7 or restoring pRb or P53 activity. Although experiments in vitro using antisense messages to complex E6/E7 proteins have been encouraging, it is a big jump from cells in tissue culture to an intact organism. However, as knowledge is power and as knowledge of cervical cancer and its etiology and molecular basis has exploded, it is not unrealistic to believe that finding molecular solutions to HPV-induced cellular problems is possible (Arends, *et al*. 1990).

2.3.8. Methods of diagnosis of cervical cancer and detection of HPV:

2.3.8.1. Clinical Diagnosis:

Early diagnosis of cervical cancer can be extremely challenging because of the three factors:

- The frequently asymptomatic nature of early-stage disease.
- The origin of some tumors from within the endocervical canal or beneath the epithelium of the ectocervix, making visualization on spectrum examination impossible.
- The significant false-negative rate for Pap smears, even in women having regular screening (Berek and Hacker, 2005).

Early invasive carcinoma of the cervix may be either asymptomatic or associated with trivial symptoms leading to a delay in diagnosis. Therefore, it is necessary to subject every married woman, irrespective of her age, to annual physical and cytological examinations (Mehta and Bansal, 2004).

2.3.8.1.1. Symptoms:

The common symptoms are intermenstrual bleeding, postcoital bleeding, postmenopausal bleeding, undue prolongation of the menstrual period and leucorrhoea (Mehta and Bansal, 2004; Berek and Hacker, 2005). However these complaints are often considered inconsequential, since they are also present in chronic cervicitis, cervical polyps and a number of other non-malignant conditions (Mehta and Bansal, 2004). Unlike endometrial cancer, which usually bleeds early, cervical cancer often is asymptomatic until quite advanced in women who are not sexually active. Large tumors become infected, and a vaginal discharge, sometimes malodorous, may occur before the onset of bleeding. In very advance cases, pelvic pain, pressure symptoms pertaining to the bowel or bladder, and occasionally vaginal passage of urine or feces may be presenting symptoms (Berek and Hacker, 2005). It is thus easy to appreciate the overwhelming value of cervical cytology in the diagnosis of early cervical cancer (Mehta and Bansal, 2004).

2.3.8.1.2. Signs:

Physical examination should include palpation of the liver, supraclavicular, and groin nodes to exclude metastatic disease (Mehta and Bansal, 2004; Berek and Hacker, 2005). On speculum examination, the primary lesion may be exophytic, endophytic, ulcerative, or polypoid. If the tumor arises beneath the epithelium or in the endocervical canal, the ectocervix may appear macroscopically normal. Direct extension to vagina is usually grossly apparent, but the infiltration may be subepithelial and suspected only on the basis of obliteration of the vaginal fornices or the presence of apical stenosis. In the later situation, it may be difficult to visualize the cervix. On palpation, the cervix is firm (except during pregnancy) and usually

expanded (Berek and Hacker, 2005). The size of the cervix is best determined by rectal examination, which is also necessary for the detection of any extension of disease into the parametrium (Mehta and Bansal, 2004; Berek and Hacker, 2005).

2.3.8.2. Radiology:

Chest X-ray is essential. Thirty to forth percent of patients with stage IIIB disease may have abnormal urograms (Mehta and Bansal, 2004).

2.3.8.2.1. Computed Tomography (CT):

CT has been used to help stage pelvic cancer since approximately 1975 (Berek and Hacker, 2005). CT scan is a medical imaging procedure that uses computer-processed X-rays to produce tomographic images or 'slices' of specific areas of the body. These cross-sectional images are used for diagnostic and therapeutic purposes in various medical disciplines. Digital geometry processing is used to generate a three-dimensional image of the inside of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation (Herman, 2009). In addition to the lymph nodes, a pelvic and abdominal CT scan allows an evaluation of the liver, urinary tract, and bony structures (Mehta and Bansal, 2004; Berek and Hacker, 2005). Compared to lymphangiography, CT is less time consuming less technically difficult, and provides more information (Berek and Hacker, 2005).

2.3.8.2.2. Ultrasonography:

Abdominal and pelvic ultrasonography is cost effective for assessing the extent cervical carcinoma. Retroperitoneal nodes and abdomino-pelvic masses can be diagnosed. It is inaccurate in predicting parametrial extension. Transvaginal ultasonygraphy is superior in providing information about tumor volume and

parametrial spread (Mehta and Bansal, 2004). As with CT ultrasonography is unable to differentiate between benign and malignant enlargement of lymph nodes, but it has the advantage of being less costly and less time consuming, and avoiding exposure to radiation (Berek and Hacker, 2005).

2.3.8.2.3. Magnetic Resonance Imaging (MRI):

MRI, which has been used since the early 1980s, has high-contrast resolution and multiplanar imaging capability and is a valuable modality for determining tumor size, degree of stromal penetration, vaginal extension, parametrial extension, and lymph node status, makes it a useful adjunct to clinical evaluation in treatment planning. MRI is also appropriate for the evaluation of pregnant patients because no risk to the fetus (Berek and Hacker, 2005). For cervical carcinoma it is important to know the size, extent of local disease and if the parametrium is involved. MRI has better soft tissue resolution to achieve these objectives (Mehta and Bansal, 2004).

2.3.8.2.4. Positron Emission Tomography (PET):

This imaging technique has been available in some centers since the mid-1990s. It depends on metabolic, rather than anatomic, alteration for the detection of disease. PET use radioneuclides, which decay with the emission of positrons (positivity charge particles). Because cancer cells are avid users of glucose, a radionuclide- labeled analogue of glucose, 2-fluoro-2-deoxy-D-glucose (FDG), can be used to detect sites of malignancy by identifying sites of increased glycolysis. The PET scan has the potential more accurately to delineate the extent of disease, particularly in lymph nodes that are not enlarged and in distant sites that are undetectable by conventional imaging studies (Berek and Hacker, 2005).

2.3.8.3. Cytology:

Conventional Papanicolaou smears (Pap smears) or liquid based methods, such as ThinPrep (Cytyc Corporation) or SurePath (BDdiagnostics- Tripath), are typically used for cervical cytology (Shahabi, *et al*. 2010). Cytology has been applied to the diagnosis of HPV infection of the penis, vulva, and anus, but cervical cytology, in the form of Pap smear remains the most extensively used. Cells can be collected by washings, but swabbing or scraping is standard. Typically the Ayre spatula is used to scrape the exodocervix, and a brush to sample the endocervix (Douglas, *et al*. 2004). The ability of the conventional Pap and liquid-based cytology to detect pre-invasive lesions is reported to be similar. The sensitivity and specificity of the conventional Pap smear are 30-87% and 70-100%, respectively, for the detection of the cervical cancer and its precursors (CIN1-3) (Shahabi, *et al*. 2010).

The presence of malignant cells in a background of necrotic debris, blood, and inflammatory cells is typical of invasive carcinoma. Differentiation between squamous and glandular cells is usually possible except for poorly differentiated lesions. The false-negative rate for Pap smears in the presence of invasive cancer is up to 50%, so a negative Pap smear should never be relied on in a symptomatic patient (Berek and Hacker, 2005). Koilocytes and dyskeratocytes (small, much keratinized squamous cells with orange cytoplasm and nucleus atypia) are hallmarks of cervical HPV infection. Cytology is at least 10 times less sensitive than polymerase chain reaction (PCR) for the diagnosis of HPV infection and should not be used for these purposes (Douglas, *et al*. 2004).

2.3.8.3.1. Conventional Papanicolaou smear:

Pap smear must be taken with spatula, with or without an endocervical brush, according to the type of the cervix. The spatula should make good contact with the transformation zoon. If a single device dose not accommodates the anatomy of the woman's cervix, two separate devices must be used. Once taken, the sample should be promptly applied to the glass slide using a side-to-side motion and fixed with alcohol to prevent air drying. The slide should be accurately pre-labeled, and sent to the laboratory in a shatterproof box. When followed diligently, these simple steps produce substantial improvement in the accuracy of the test. Considerable effort has been devoted to improving laboratory testing procedures, particularly using computerizing screening methods to support laboratory personnel (Shahabi, *et al*. 2010).

2.3.8.3.2. Liquid-based methods:

Involve placing the cells collected into a vial of preserving liquids. The ThinPrep system involves collecting samples from the cervix in a similar manner to that used in the Pap smear except that plastic extended-tip spatulas are recommended. After the combined ectocervical and endocervical sample is obtained, the spatula is swirled vigorously in the alcohol-based preservative solution and then discarded. If a separate endocervical brush is used to obtain an endocervical sample, the brush is fully inserted into the endocervical canal and rotated a quarter or half turn in one direction. The brush is then quickly placed in the preservative solution to prevent drying and then rinsed in the solution by rotating the brush in the solution ten times while pushing against the vial wall. The brush is also swirled vigorously in the

solution to release any remaining material and then the brush is discarded (Shahabi, *et al.* 2010).

2.3.8.3.3. Fine-Needle Aspiration Cytology (FNAC):

If pelvic or abdominal masses or enlarged lymph nodes are detected during physical examination or imaging studies, FNA may be performed under CT or ultrasonic guidance. The procedure is performed under local anesthesia and is free of major complications, even in the presence of clotting problems or perforation of a hollow viscus. The reported accuracy for abdominopelvic nodes ranges from 74% to 95%. Only appositive cytologyic diagnosis should be used as a basis for therapeutic decision making (Berek and Hacker, 2005)

2.3.8.4. Direct visual inspection:

Direct visual inspection (DVI) with acetic acid and visual inspection with Lugol's iodine used to highlight precancerous lesions so can be viewed with the "naked eye" (Sherris, *et al*. 2009). Also can be employed in low-resource settings as an alternative method of cervical cancer screening. Cervicoscopy, speculoscopy and cervicography are different means of visually inspecting the cervix, and each provides a reasonable alternative to the typical screening regimen. In the presence of normal cytology results, a visible lesion on the cervix that is raised, friable, or has the appearance of condyloma should be biopsied regardless of the smear diagnosis. Conversely, the sensitivity of colposcopy is not as good as was once believed. A decade ago, still for many current practitioners, colposcopy was considered the 'gold standard'. However prospective investigations have revealed the limitation of colposcopic impression and single-directed biopsies (Shahabi, *et al*. 2010). As a screening test, DVI performs

equal to or better than cervical cytology in accurately identifying pre-cancerous lesions (Sherris, *et al*. 2009).

2.3.8.5. Histopathology:

Histology is the gold standard for confirming diagnosis of HPV disease, and is the most important among the laboratory resources available to the clinician. Histologic criteria allow the diagnosis of the different HPV diseases. They are neither absolute nor easy to define, and subjectivity and experience contribute to differences in interpretation (Douglas, et al. 2004).

2.3.8.5.1. Biopsy:

Any obvious tumor growth or ulceration should undergo office punch biopsy or diathermy loop excision for histologic confirmation (Mehta and Bansal, 2004; Berek and Hacker, 2005). Any cervix that is unusually firm or expanded should also undergo biopsy and endocervical curettage (ECC) (Berek and Hacker, 2005). If the patient has a normal-appearing cervix but is symptomatic, or has an abnormal Pap smear, colposcopy should be performed (Mehta and Bansal, 2004; Berek and Hacker, 2005). If a definitive diagnosis of invasive cancer cannot be made on the basis of an office biopsy, diagnostic conization may be necessary (Berek and Hacker, 2005). For polypoidal or ulcerated lesions, a punch biopsy is adequate to arrive at a diagnosis. Cervical conisation is done to assess the extent or depth of invasion. In CIN, it is a diagnostic as well as a therapeutic procedure. A cold knife cone biopsy defines stromal invasion in patients with severe dysplasia or carcinoma *in situ*. The presence of such an extension may be missed in punch biopsies. The cone biopsy should include the entire squamocolumnar junction and the lower half of the endocervical canal. A colposcopically directed conisation can lead to better delineation of excised

margins. Involved margins and positive curettage above the cone bed may indicate a higher risk of recurrence. Proper haemostasis is mandatory by cautery, sutures or haemostatic agents. Patients should follow vaginal abstinence for at least two weeks. Other complications of cone biopsy include cervical incompetence (due to damage to the sphincter) and cervical stenosis (Mehta and Bansal, 2004).

2.3.8.6. Culture and Serological assays:

HPV cannot be isolated in cell culture because it requires cell differentiation to complete its replication cycle, so the serological assays have only limited accuracy (Schiffman, *et al*. 1995, Dillner, 1999).

There are no serologic assays satisfactory for the clinical diagnosis of HPV infection. The most consistently sensitive assays are enzyme linked immune assay (ELISA) enzyme immunoassay (EIA), and western blot and radioimmunoprecipitation assay (Douglas*, et al.* 2004, Dillner, 2006). They cannot detect much more than 50% of infected subjects if they are to retain a high specificity (Douglas, *et al*. 2004). Advantages of serology include the ability to measure current and past infections and the allowance for retrospective studies on serum blanks when tissue samples are not available (Coutee, *et al*. 1997). Serology is a useful tool for epidemiological studies and vaccine trials, but is of limited value for clinical diagnosis (Douglas, *et al*. 2004; Dillner, 2006).

2.3.8.7. Electron microscopy:

Transmission electron microscopy is of little use in the diagnosis of HPV lesions, but may reveal the presence of intranuclear viral particles that are typically organized in crystalline arrays or pseudoarrays (Douglas, *et al*. 2004). Non enveloped icosahedral viral particles can be demonstrated in productive HPV infections by

electron microscope, but genotyping cannot be done with electron microscopy (Swan and Tucker, 2003).

2.3.8.8. Immunohistochemistry (IHC):

Immunohistochemistry refer to the process of detecting antigens (e.g., proteins) in cells of tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara, 2005). IHC staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors, also it can be helpful in predicting disease prognosis, and directing its plan of therapy (Helal, 2005). Immunohistochemistry has proved a very useful tool for the etiological diagnosis of microbial infections in tissue samples, including viruses such as cytomegalovirus, measles, hepatitis B and HPV. However, the expression of HPV markers and surrogate markers of HPV infection can be simply estimated by Immunohistochemistry (IHC). Diverse antigens can be used including virus related (capsidic antigens, E5-E6-E7 proteins), and virus induced and/or altered host proteins ($p16^{INK4a}$, pRb, Cyclin proteins, p-53) (Pannone, et al. 2011). Several authors have stressed that a hallmark of the presence of HPV in cancer could be found in p16 nuclear or cytoplasmic overexpression, so that p16 could be considered a useful surrogate marker for HPV (Gillespie, et al. 2009; Goon, et al. 2009). Monoclonal antibodies directed to L1 capsid protein of high risk papilloma virus (HR-HPV) were designed for specific immunohistochemical (IHC) detection of HR-HPV infections in intra -epithelial neoplasia (Sapp*, et al*. 1994). Avidin biotin technique which was applied in this study is one of the indirect methods depend on the affinity of avidin to biotin. Avidin is a glycoprotein extracted from streptoavidini, which have a four identical subunit binds one molecule of biotin. Biotin is low molecular weight vitamin,

which is easily conjugated to antibodies. This property makes this technique highly sensitive (Bancroft, *et al*. 1996). Antigen detection by immunocytochemistry by the use of polyclonal antibodies against the late structural L proteins on biopsies can confirm the presence of HPV-induced morphological changes, but the test is relatively insensitive and this technique has been replaced by in situ hybridization (Swan and Tucker, 2003). Therefore, one of the aims of this study was to assess role of IHC for detection of HR-HPV subtypes as compared to molecular identification.

2.3.8.9. Molecular biology:

2.3.8.9.1. Nucleic acid detection assays:

HPV-DNA can be detected in cervical smears and biopsy specimens by various methods, of which in-situ hybridization is complementary to cytology. Direct detection of HPV genomes as well as transcripts also can be achieved with hybridization procedures that include Southern and Northern blots, dot blots, in-situ hybridization, signal-amplification molecular technology (Hybrid Capture assay), and DNA sequencing (Molijn, *et al.* 2004). Several methods have been developed for this purpose such as PCR and restriction fragment length polymorphism (PCR-RFLP), hybridization analysis and the direct sequence analysis of PCR product (Molijn, *et al*. 2004).

2.3.8.9.2. In situ hybridization:

In situ hybridization assays for HPV DNA can provide data on the presence of HPV in different cells, but have limited sensitivity for certain HPV genotypes and cannot demonstrate oncogene transcription. Viral oncogene expression can be demonstrated by PCR technique, but this dose not provides information about the viral load and the distribution of HPV DNA (Gillison, 2006). In situ hybridization on

biopsies correlates the detection of HPV-DNA with histopathology. The presence of HPV-induced histologically equivocal lesions in biopsies can be confirmed by in situ hybridization. In situ techniques are more sensitive but need refinement (Coutlee*, et al*. 1997). PCR combined with in situ hybridization can detect HPV- infected cells with low viral loads, and can also elucidate the distribution of HPV DNA within the tumor (Miller, *et al*. 1994). Filter-based hybridization assays have been used mainly with research laboratories. Southern blots are fastidious and lengthy procedures that require several steps for the processing of samples and assay completion, and not readily applicable to routine diagnostic laboratories, often required large amount of sample DNA and use radioactive probes. Once considered the gold standard for HPV research, Southern blot has now been replaced in research laboratories by PCR assays (Lorincz, 2001).

2.3.8.9.3. Signal amplification DNA-based assays (The Hybrid Capture System) (HC):

Signal amplification tests can detect lower quantities of DNA than direct methods by amplifying and the detection signal without modifying the initial amount of nucleic acids contained in samples (Coutlee, *et al*. 1997). The HC assay has been used in many studies, and the second generation HCII version of the assay is the widely used in clinical diagnostic laboratories (Wright *et al*. 2002). The sensitivity of Hybrid Capture to detect high-grade lesions in some studies reached (90%) (Wright, *et al*. 2002).

2.3.8.9.4. Polymerase chain reaction (PCR):

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

thousands to millions of copies of a particular DNA sequence, and developed in 1983 by Kary Mullis (Bartlett, *et al*. 2003). PCR is the most powerful tool for the epidemiological investigation of HPV infection or cervical cancer (Coutlee, *et al*. 1997), and now it is the gold standard test for HPV research in which the natural history of HPV infection has been more clearly defined with PCR (Coutlee, *et al*. 1997). PCR also permits variant analysis of HPV types and allow for the discovery and characterization of novel HPV types and subsequent typing in accomplished on filters by hybridization with type-specific oligonucleotide probes (van Doorn, *et al*. 2002). PCR is the most widely used target amplification method, using a thermocycling process and employing oligonucleotide primers flaking the region of interest to amplify DNA in the presence of a thermostable DNA polymerase, two approaches for detection of HPV-DNA by PCR are relevant, type specific PCR versus broad-spectrum PCR, type specific primers diagnosed to amplify exclusively a single HPV genotype can be used, but to detect the presence of HPV-DNA in a single sample, multiple type-specific PCR reactions must be performed separately. Real-time PCR can also be used to detect HPV-DNA. Type-specific PCR primers can be combined with fluorescent probes for real-time detection (Molijn, *et al*. 2004).

2.3.8.9.4.1. Type-specific PCR:

Type-specific PCR assays are based on the sequence variations present in the E6 and E7 genes of HPV subtypes. Fourteen type-specific PCRs for high risk HPV types (HPV-16,-18,-31,-33,-35, -39, -45, -51,-52,-56, -58,-59, -66 and 68) that target approximately 100 bp in the E7 ORF have been developed (Walboomers, *et al*. 1999).

2.3.8.8.4.2. Real-time PCR:

Quantification of viral HPV-DNA in clinical specimens could improve the clinical utility of HPV detection methods (Coutlee, *et al*. 1997). Quantitative PCR assays require a control for amplification efficiency and sample inhibition. Real-time PCR assays provide accurate measurements of the initial copy number of target DNA contained in samples by continuously measuring the increments of fluorescence released during the amplification reaction (Swan and Tucker, 2003). Several formats can be used to perform real-time amplification and detection of HPV-DNA assay based on hydrolysis probes (Taqman assays); assays based on fluorescence energy transfer (SYBR green); and finally, assays using molecular beacons (Coutee, *et al*. 1997).

2.3.10. Management of cervical cancer:

2.3.10.1. Prevention:

2.3.10.1.1. Primary prevention:

Primary prevention of a disease requires warding it off before the pathogenic process can occur. In the case of cervical cancer, this would require that infection of the cervix with HPV (a necessary although not sufficient cause of cervical cancer) be prevented. It could be achieved either by complete abstinence from sexual activity or with a vaccine (Lynette and Rengaswamy, 2006). Human papillomavirus (HPV) vaccines have the potential to reduce cervical cancer incidence and mortality, particularly in the parts of the developing world that bear the greatest burden of disease (Tracy, *et al.* 2014). Two prophylactic HPV vaccines are available; both vaccines are based on virus-like particles of the L1 capsid protein, and are highly efficacious and immunogenic if given before exposure to HPV (Dochez, *et al*. 2014).

The Food and Drug Administration has approved a quadrivalent, virus-like particle vaccine that consists of noninfectious peptide particles of HPV-6, HPV-11, HPV-16, and HPV-18 (Gardasil; Merck & Co, Inc, Whitehouse, NJ) When administered in a series of 3 immunizations in the 6 months before initiation of sexual activity, the vaccine confers a high level of immunity to these serotypes. The vaccine is currently recommended for females aged 9 to 26 years and is expected to markedly reduce the incidence of high-risk HPV infections and cervical dysplasia and to reduce the incidence of invasive cervical cancer in future generations of young women. A second bivalent vaccine (Cervarix; GlaxoSmithKline, Brentford, Middlesex, UK), which is directed at prevention of HPV-16 and HPV-18, is reported to show substantial cross-protection for HPV-31 and HPV-45 (Harry, et al. 2007). To increase the protection, the development and testing of a nine-valent prophylactic HPV vaccine (HPV6/11/16/18/31/33/45/52/58) is being undertaken. Research is also directed towards therapeutic vaccines and the development of a prophylactic L2 vaccine (Dochez, *et al*. 2014).

2.3.10.1.2. Secondary prevention:

Secondary prevention involves early identification of pre-cancerous lesions and stops the progression of disease once it has already started by cytology based Papanicolaou (Pap) smear screening, and the treatment of such lesions can help in preventing cervical cancer (Lynette and Rengaswamy, 2006; Lim, *et al*. 2014). Cervical screening in combination with immunization of adolescents and young women may be an effective strategy for the prevention of cervical cancer (Lim, *et al.* 2014). The Pap smear has been used for more than 50 years in the United States and developed nations for early detection of cervical cytologic abnormalities seen with

cervical dysplasia and cervical carcinoma. Screening has resulted in a significant reduction in both incidence and deaths due to invasive squamous cell cervical cancer. The cytologic screening of cervical epithelium has improved throughout the years, and liquid-based cytologic screening and reflex HPV testing at 3-year intervals in sexually active women have been shown to have a sensitivity of 80% and a specificity of 95%. Current recommendations advise initiating Pap testing 3 years after first intercourse with continued screening at 1- to 3-year intervals, depending on age, risk factors, and the specific guideline being used. Although Pap smear screening has significantly reduced incidence and mortality in the developed nations, it is generally not available in developing nations, where cervical cancer remains a major public health problem for young women (Harry, *et al*. 2007).

2.3.10.2. Treatment:

The treatment of invasive cervical cancer can include surgery, radiation therapy, chemoradiation therapy, or chemotherapy (Harry, *et al*. 2007), depending on many factors including histological types and degree of differentiation. Degree of differentiation is an important consideration in determining the prognostic outcome (Eriba, *et al*. 2013). Unlike more indolent diseases, the progression-free survival closely approximates overall survival, reflecting the poor salvage rates for recurrent or metastatic disease in this malignancy. In addition, cervical cancer is most commonly diagnosed with disease limited to the pelvis; thus, appropriate locoregional therapy at the initial diagnosis is necessary for optimal outcomes. For patients with stage IA1, IA2, and IB1 disease, the probability of surgical cure with hysterectomy (simple or radical, depending on tumor characteristics) and pelvic lymphadenectomy exceeds 90%, and these patients are considered primary surgical candidates, while for

patients with bulky stage IB2, IIA, IIB, IIIA, IIIB, and IVA disease, best results are obtained with external beam radiotherapy to the whole pelvis with additional intracavitary radiation to deliver a sterilizing dose to the tumor, plus concurrent cisplatin-based chemotherapy.women with stage IA1, IA2, and IB1 disease if fertility preservation is desired (Harry, *et al*. 2007).

Adenocarcinoma is relatively radioresistant. Surgical treatment is preferred over radiation therapy. Sarcoma botryoides is no more treated by total pelvic exenteration as in the past. Local excision followed by radiation and chemotherapy is preferred. Leiomyosarcoma is treated by hysterectomy. Lymph nodes dissection is not indicated. Small cell carcinomas are best treated with surgery; however, they are usually diagnosed at advanced stages and may be inoperable. In small series, small cell cancer has demonstrated a very bad prognosis. Glassy cell carcinoma behaves aggressively and carries a bad prognosis; these patients are treated with radical hysterectomy and radiation (Mehta and Bansal, 2004).

2.3.10.3. Prognosis:

A woman's prognosis is determined by stage, nodal status, tumor volume, depth of cervical stromal invasion and histological types and grades (Shahabi, *et al*. 2010). Persistent or recurrent carcinoma of the cervix is a discouraging clinical entity of the clinician, with a 1-year survival rate between 10% and 15%. Treatment failures are, as expected stages of the disease; therefore, most patients are unlikely candidates for a second curative approach with radical pelvic surgery. Cases of curative therapy applied to isolated lung metastases or lower vaginal recurrences are reported but occur rarely (Disaia and Creasman, 2002). The histological type of the tumor is less important for prognosis than is the staging at diagnosis. Microinvasive

carcinoma show minute foci of very superficial invasion, only detected histologically, and have a very good prognosis after local excision. Invasive carcinomas are staged according to the degree of local invasion, and survival is related to stage. Invasion of paracervical and external iliac nodes occur early (Stevens, *et al.* 2009). In general, cervical squamous carcinoma has a better prognosis than adenosquamous carcinoma, stage for stage. In addition, adenocarcinoma tends to be diagnosed later and therefore at a more advanced stage, which worsens the prognosis (Shahabi, *et al*. 2010).

2.3.11. Cervical cancer in Yemen:

Yemen has a population of 5.58 million women ages 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 370 women are diagnosed with cervical cancer and 206 die from the disease. Cervical cancer ranks as the 3rd most frequent cancer in women in Yemen, and the 10th most frequent cancer among women between 15 and 44 years of age. Data is not yet available on the HPV burden in the general population of Yemen (Castellsague, *et al.* 2007). However, in Western Asia, the region Yemen belongs to, about 2.2% of women in the general population are estimated to harbour cervical HPV infection, and 66.7% of invasive cervical cancers are attributed to HPVs 16 or 18. Incidence of cervical cancer in Yemen is 1.4 per 100,000 women per year in 2008 (Maha Abdul-Aziz, 2012).

Chapter three

Materials and Methods

3.1. Study design:

This is a retrospective analytical case control study which aimed to determine the molecular genotyping and immunophenotyping of high risk HPV types among Yemeni women diagnosed with cervical cancer.

3.2. Study area:

This study was conducted in Yemen [in Sana'a (the capital of Yemen), Aden (the commercial and economical capital of Yemen - the second city in Yemen), and Hadhramout (one of the main governorate in Yemen)]. In these three governorates there are the only three national cancer centers in Yemen and all cancer patients come from different governorates of Yemen are referred to diagnose and take treatment. The study was conducted during the period from January 2012 to January 2015.

3.3. Sample size:

Sample size was calculated by the following formula:

$$\text{Sample size (n)} = \frac{(1.96)^2\, pb}{D^2}$$

(1.96) = fixed error, p= past prevalence = 50 because there is no previous study, b= 1- p (1=100%), D = expected error (1- 13) the best 5 or 10 in this study 10 is used, so from this formula the sample size = 96, then it was completed up to two hundred samples for both cases and controls.

3.4. Materials:

Formalin-fixed paraffin-embedded tissue blocks and data about the related patients were collected during this study.

3.5. Sample collection:

One hundred and fifty samples with cervical malignant lesions (as cases) which included low-grade CINs (CIN 1), high grade CINs (CIN2, 3), invasive squamous cell carcinomas (ISCCs) and adenocarcinomas, and fifty samples with non neoplastic lesions (as controls) from patients who had undergone hysterectomy for any cause other than cervical cancer were collected in this study. All specimens and pertinent data were obtained from the national cancer centers, different hospitals and private histopathology laboratories in the three governorates mention above during the period from 2009 to 2013.

3.6. Sample preparation:

3.6.1. Histopathological tissue preparation:

Sections of 3 micron thickness were obtained from each formalin-fixed paraffin-embedded tissue block by using rotary microtome and stained using haematoxyline and eosin (Mayer's technique) to confirm the histopathological diagnosis. All sections were dewaxed in hot plate oven and cleared in two changes of xylene for two minutes in each, hydrated through descending grades of ethyl alcohol (100%, 90%, 70%, 50%) and water two minutes for each, stained in Mayer's haematoxyline for seven minutes, then washed and blued in running tap water for ten minutes, stained in eosin for three minutes, washed in distilled water after that hydrated through ascending grades of ethyl alcohol, cleared in xylene and finally mounted in DPX mountant (Bancroft and Gamble, 2002).

3.6.2. Immunohistochemical tissue preparation:

Sections of 3 micron thickness were obtained from each formalin-fixed paraffin- embedded tissue block using rotary microtome on silanized coated slides and were retrieved by water bath heat retrieval technique, then immunostained using avidin biotin technique to detect the HPV by the use of monoclonal mouse anti-human papillomavirus clone (K1H8) from Dako company to demonstrate HPV type 6,11,16,18,31,33,42,51,52,56 and 58. All sections on silanized coated slides were dewaxed in hot plate oven and cleared in two changes of xylene two minutes for each, hydrated through descending grades of ethyl alcohol (100%, 90%, 70%, 50%) and finally to distilled water, 2 minutes for each change. The sections were boiled in the Target Retrieval Solution of Dako (citrate buffer solution pH 6) in a water bath at 95°c for 30 min, then left to cool at room temperature and washed three times with phosphate buffer saline (PBS). 3% hydrogen peroxide in methanol was added to each section for 15 min to block endogenous peroxidase activity, and then washed three times with PBS (pH7.4) for 3 minutes, then the diluted monoclonal mouse anti-human papillomavirus clone (K1H8) was added to each slide for 30 minutes, washed in PBS for 3 min., then treated with biotinylated link for 15 minutes, washed in PBS for 3 min, treated in conjugated streptavidin, washed in PBS for 3 min, then treated with 3,3- diaminobenzidinetetrahydrochlorate (DAB) for 10 minutes, washed in PBS for 3 minutes, then stained in Mayer's haematoxyline as counter stain for one minute, then washed and blued in running tap water, hydrated, cleared and mounted in DPX(as described by Dako company) .

Positive and negative controls from known positive and negative samples were treated as the samples.

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

From each paraffin block, small sections of 30-50 microns thick were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut using new gloves and new disposable microtome blade; knife holder was cleaned with xylene and rinsed with alcohol between sectioning of the blocks. All forceps and utensils used were also cleaned in this manner.

3.6.3.1. DNA Extraction:

DNA was extracted according to the steps described in DNA extraction kit that was purchased from Sacace biotechnologies-Casera –Italy, as follows:-

Xylene was added to each tube up to cover the tissues for dewaxing, the tubes were vortexed vigorously and incubated at 37°C for about 30 min., vortexed then centrifuged at 800g per 5 min., the supernatant was carefully discarded from each tube. Xylene was added again to each tube vortexed vigorously incubated at room temperature for 3 min., and centrifuged for 5 min. at a maximum speed (16000g), all supernatant was pipetted off and after that the sections were re-hydrated with descending grades of ethanol (100%, 75%, 50%, 25%) and sterile water, vortexed and centrifuged for 3 min. at a maximum speed after each washing, the water was discarded from the pellet, then 300 micro liters (µl) of Lysis Solution were added to each tube and vortexed vigorously, incubated for 5 min at 65°C, centrifuged for 5 min at a maximum speed (16000 g.) and the supernatant was transferred into a new tube for DNA extraction. The sorbent was vortexed vigorously and 25 µl from it was added to each tube, vortexed for 5-7 sec and incubated for 3 min at room temperature, vortexed again and incubated for 3 min. at room temperature, centrifuged for 30 seconds at 5000g and the supernatant was carefully removed and discarded from

each tube without disturbing the pellet by using a micropipette in which the tips were changed between the tubes. After that 300 µl of Washing Solution 1 was added to each tube, vortexed vigorously and centrifuged for 30 sec. at 8000g. The supernatant was then removed and discarded from each tube, then 500 µl of Washing Solution 2 was added to each tube, vortexed vigorously and centrifuged for 30 sec. at 8000g. the supernatant was removed and discarded from each tube, 500 µl of Washing Solution 2 was added again to each tube, vortexed vigorously and centrifuged for 30 sec. at 8000g, the supernatant was removed and discarded from each tube, and incubated with open cap for 5 min at 65°C, the pellet was resuspended in 50 µl of DNA-eluent, incubated for 5 min at 65°C and vortexed periodically, centrifuged for a min. at 12000g, the supernatant contains DNA was ready for amplification, then the processed samples were stored at –20°C (as described by Sacace biotechnologies company).

3.6.3.2. Polymerase chain reaction (PCR):

3.6.3.2.1. Amplification of HPV:

Type specific primers (primer for HPV 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66) were used to detect HPV 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 DNA in cervical benign and malignant samples. Amplification was performed according to HPV High Risk Typing Kit from Sacace biotechnologies- Casera –Italy, as follows:

Required quantity of tubes for samples and controls were prepared (blue for PCR-mix-1 “16-35”, rose for PCR-mix-1 “18-59” and green for PCR-mix-1 “52-66”), one new tube was prepared for each PCR-mix-1 and for each sample 5*(N+1) µl of PCR-mix-1, 10*(N+1) of 2,5 x buffer and 0,5*(N+1) of TaqF Polymerase were added , 15 µl from the reaction mix was added into each sample tube then to appropriate tube

10 µl of DNA sample obtained after sample preparation was added, 1 drop (25 µl = of Mineral Oil) was added to each sample tube.

Controls were Prepared as following:

Negative Control: 10 µl of DNA-buffer was added to the tube labeled Cnegampl.

Positive controls:

Of PCR-mix-1 “16-35” 10 µl of each HPV 16, 31, 33 and 35 DNA (C+) was added to the 4 labeled blue tubes;

Of PCR-mix-1 “18-59” 10µl of each HPV 18, 39, 45 and 59 DNA (C+) was added to the 4 labeled rose tubes;

Of PCR-mix-1 “52-66” was 10 µl of each HPV 52, 56, 58 and 66 DNA (C+) was added to the 4 appropriate green tubes;

All tubes were closed and transferred into the thermocycler and started the following program:

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

Thermocyclers with active temperature adjustment. “PE 2400” (Perkin Elmer), Omn-E (Hibaid) and other.			
Step	**Temperature °C**	**Time**	**cycles**
1	95 °C	Pause	
2	95 °C	15 min	1
3	95 °C	30 sec	42
	63 °C	30 sec	
	72 °C	40 sec	
4	72 °C	1 min	1
5	10 °C	Storage	

3.6.3.3. Gel-electrophoresis:

The PCR products were visualized in 3% Agarose gel with 0.5 µg/ml Ethidium bromide stain. The gel was prepared by dissolving 3 grams of agarose powder in 100 ml of 1X TBE buffer and heated at 65°C until the agarose completely dissolved then left to cool at room temperature and 2µl ethidium bromides was added. The comb was placed appropriately in the electrophoresis tray and then gel was slowly poured and left to set for 30 min for solidification. Ten µl of 100 base pairs (bp) DNA ladder and PCR products were loaded on the gel. Gel-electrophoresis was performed at 120V and 36 Am for 40 minutes. Pictures were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

3.7. Results Interpretation:

All quality control measures were adapted during sample preparation and processing for the assessment of histopathological, immunohistochemical and molecular results.

Each slide for histopathology and immunohistochemistry was screened and confirmed by expert pathologist and histotechnologist.

Interpretation of PCR results was done according to manufacture HPV High Risk Typing Kit (from Sacace biotechnologies- Casera –Italy) manual. The PCR product length for HPV 16 should be 325 bp; HPV 31, 520 bp; HPV 33, 227 bp; HPV 35, 280 bp; HPV 18, 425 bp; HPV 39, 340 bp; HPV 45, 475 bp; HPV 59, 395 bp; HPV 52, 360 bp; HPV 56, 325 bp; HPV 58, 240 bp and HPV 66 should be 304 bp.

3.8. Statistical analysis:

Data obtained from this study were analyzed by using statistical package for social science software (SPSS). Frequency, mean and Chi-Square test were used to state the significance of results, P value < 0.05 was considered statistically significant.

3.9. Ethical consideration:

Before the study conducted the proposal of the study was ethically approved by ethical committee of the Sudan University of Science and Technology. Then inform consents were obtained from the directors (managers) of the national cancer centers, hospitals and private histotechnology laboratories from which the samples and data used in this study were obtained.

Chapter four

Results

A total of 150 cases (patients diagnosed with cervical cancer) and 50 controls (patients with non neoplastic lesions who had undergone hysterectomy for any cause other than cervical cancer) were included in this study and investigated for the presence of HR-HPV infection by immunohistochemistry techniques and molecular biology technique (PCR). The age distribution was relatively similar between cases and controls with a range of 21 to 75 years and mean age of 46.73 years. in general, most of the study subjects were in the age group 41–50 years representing 88/200 (44%) followed by age group 51-60, 31-40, 61-70, 21-30 and 71-80 representing 40/200 (20%), 39/200 (19.5%), 19/200 (9.5%), 13/200 (7.5%) and 1/200 (0.5%) respectively, as indicated in Table 1. The distribution of the study population by region is shown in Table (1), most of cases were from Al-Janad region representing 52/200 (26%) followed by Aden, Azaal, Hadhramout, Tohama and Saba'a regions representing 45/200 (22.5%), 40/200(20%), 30/200 (15%), 24/200 (12%) and 9/200 (4.5%) , respectively.

As indicated in Table (1), squamous cell carcinoma (SCC) was the most common among cases representing 80/150 (53.3%) followed by adenocarcinoma, CIN 3, CIN1, CIN 2, adenosquamous carcinoma, clear cell carcinoma and glassy cell carcinoma representing 19/150 (12.7%), 16/150 (10.7%), 15/150 (10%), 13/150 (8.7%), 3/150 (2%), 2/150 (1.3%) and 2/150 (1.3%), respectively. In comparison between the detection of HR-HPV by immunohistochemistry techniques and PCR as shown in Table (2, 3) immunohistochemically HPV was detected in 78/150 (52%) of cases and 6/50 (12%) of controls while by PCR HPV types were detected in 114/150

(76%) of cases and 8/50 (16%) of controls. As shown in Table (4) PCR is better than immunohistochemistry in the detection of HPV in which by using PCR 122 cases were positive while, by immunohistochemistry there was only 84 cases that gave positive results, and by using Chi-square test the results show positively statistical significant in which the P. Value was (P=0.000). Tables (3) and (5) summarize the results of HR-HPV types among cases and controls groups in which the prevalence of HR-HPV was 76% (114/150) among cases and 16% (8/50) among control; ten HR-HPV types were detected in this study. Consequently, the risk associated with HPV infection was found to be statistically significant in which the P. Value was (P=0.000). Among the positive patients, 73% (89/122) had single type infections, and 27% (33/122) had multiple HPV types involving mainly HPV 16, HPV 18 and HPV 31. 10/33 (30.3%) were positive for HPV 16 and 18, 4/33 (12.1%) HPV 16 and 45, 3/33 (9.1%) HPV 16 and 39, 2/33 (6%) HPV 16 and 59, 2/33 (6%) HPV 16 and 58, 4/33 (12.1%) HPV 18 and 31, 2/33 (6%) HPV 31 and 45, 1/33 (3%) HPV 31 and 39, 1/33 (3%) HPV 35 and 18, 1/33 (3%) HPV 35 and 39, 1/33 (3%) HPV 35 and 59, 1/33 (3%) HPV 18 and 58, and 1/33 (3%) were positive for HPV 45 and 58, as shown in figure (1). The most common genotypes among cases were HPV 16 72/150 (48%) (51/72 as single infection, 21/72 as multiple infections), the second most common was HPV 18 31/150 (20.7%) (16/31 as single infection and 15/31 as multiple infection) followed by HPV 31 9/150 (6%), HPV 45 9/150 (6%), HPV 58 6/150 (4%), HPV 33 5/150 (3.3%), HPV 35 5/150 (3.3%), HPV 39 5/150 (3.3%), HPV 59 4/150 (2.7%) and HPV 52 1/150 (0.7%). While, among the control group HPV 16 2/50 (4%), HPV 31, 33, 35, 18, 45, and 58 each one represent 1/50 (2%) as shown in Table (5). In total, types 16 and 18 alone or in co-infection with each other were detected in 68.6% of cases.

Genotypes 31 and 45 were the third most common types. Overall, genotypes 16, 18, 31, 45, and 58 were the five most common types. HPV 31 was mainly detected as co-infection with other types. As shown in Table (6) most of SCC 35/80 (43.7%) were found in the age group 41-50, but there was no statistically significant association between the age group and type and degree of cervical cancer (P= 997). Table (7) also shows that most of the cases in the age group 41-50 24/88 (27.3%) were from Al-Janad region, but in general there was no statistically significant association between the age group and region. Tables (8 a, b, c) show that there was an association between some types of HR-HPV and age group. HPV 16 was strongly associated with age groups less than 50 years (P=0.005), HPV 58 (one case) was also associated with age group 71-80 years (P=0.000). There was no statistically significant association between other HR-HPV types and age group in this study. Tables (9 a, b, c) show the association between the HR-HPV types and type of cervical cancer in which there was a statistically significant association with HPV 16, 18, and 52 with P. Value (P=0.000), in which HPV 16 was most common among SCC and CIN3, HPV 18 was more common among adenocarcinoma while, the only one positive case of HPV 52 was adenosquamous carcinoma. Tables (10 a, b, c) show that there was no statistically significant association between any types of HR-HPV and region in this study. Also in this study there was no statistically significant to associate between geographical regions and type of cervical cancer as shown in Table (11). Table (12) shows that there was a statistically significant association between age group and the results of immunohistochemistry; most of cases that were positive by immunohistochemistry were in the younger age group, (P=0.05). There was no statistically significant association between immunohistochemistry results and

region in this study as seen in Table (13), but most of SCC, CIN2 and 3 were positive by immunohistochemistry technique, so that there was statistical significant association between immunohistochemistry results and type of cervical cancer (P=0.000) as shown in Table (14).

Table (4.1): Distribution of study population by age, region and types of cervical cancer.

Variable	Category	Case		Control		Total
		No	%	No	%	
Age	21 - 30	10	6.7	3	6	13
	31 - 40	29	19.3	10	20	39
	41 - 50	66	44	22	44	88
	51 - 60	30	20	10	20	40
	61 - 70	14	9.3	5	19	19
	71 - 80	1	0.7	0	0	1
Total		150	100	50	100	200
Region	Azaal	29	19.3	11	22	40
	Aden	34	22.7	11	22	45
	Hadhramout	20	13.3	10	20	30
	Al-Janad	39	26	13	26	52
	Tomama	20	13.3	4	8	24
	Saba'a	8	5.3	1	2	9
Total		150	100	50	100	200
Diagnosis	CIN 1	15	10	0	0	15
	CIN 2	13	8.7	0	0	13
	CIN 3	16	10.7	0	0	16
	SCC	80	53.3	0	0	80
	Adeno Carcinoma	19	12.7	0	0	19
	AdenosquamousCa	3	2	0	0	3
	Clear Cell Ca	2	1.3	0	0	2
	Glassy Cell Ca	2	1.3	0	0	2
	No Cervical Lesion	0	0	50	100	50
Total		150	100	50	100	200

Table (4.2): Immunohistochemical results among study population

		Sample		Total
		Case	Control	
Immuno Results	Pos.	78	6	84
	Neg.	72	44	116
Total		150	50	200
P. Value		0.000		

Table (4.3): PCR results among study population

			Sample		Total
			Case	Control	
PCR Results	Pos.	Multiple inf.	33	0	33
		Single inf.	81	8	89
	Neg.		36	42	78
Total			150	50	200
P. Value			0.000		

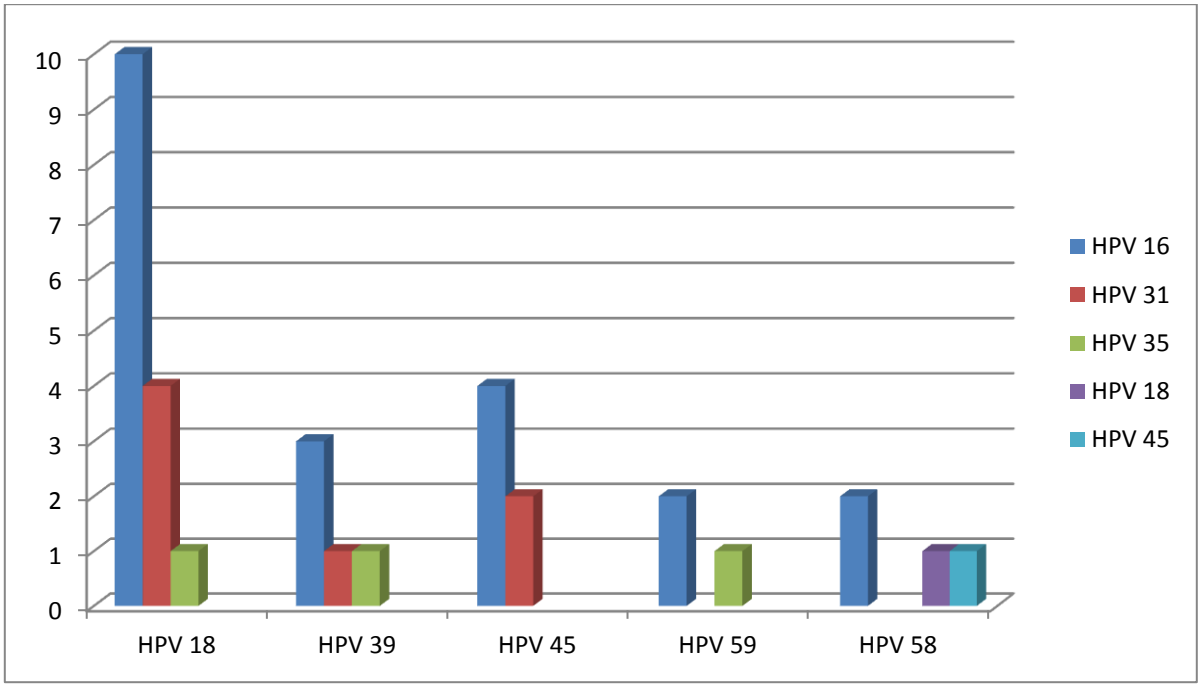


Figure (4.1): Multiple infections of some HR-HPV types

Table (4.4): Comparison of immunohistochemistry and PCR results among study population

		Immuno		Total
		Pos.	Neg.	
PCR	Pos.	81	41	122
	Neg.	3	75	78
Total		84	116	200
P. Value		0.000		

Table (4.5): Distribution of HR-HPV among study population

			Sample				Total	P. Value
			Case		Control			
			No	%	No	%		
HR-HPV types by PCR	HPV 16	Pos.	72	48	2	4	74	0.000
		Neg.	78	52	48	96	126	
	HPV 31	Pos.	9	6	1	2	10	0.261
		Neg.	141	94	49	98	190	
	HPV 33	Pos.	5	3.3	1	2	6	0.632
		Neg.	145	96.7	49	98	194	
	HPV 35	Pos.	5	3.3	1	2	6	0.632
		Neg.	145	96.7	49	98	194	
	HPV 18	Pos.	31	20.7	1	2	32	0.002
		Neg.	119	79.3	49	98	168	
	HPV 39	Pos.	5	3.3	0	0	5	0.191
		Neg.	145	96.7	50	100	195	
	HPV 45	Pos.	9	6	1	2	10	0.261
		Neg.	141	94	49	98	190	
	HPV 59	Pos.	4	2.7	0	0	4	0.243
		Neg.	146	97.3	50	100	196	
	HPV 52	Pos.	1	0.7	0	0	1	0.563
		Neg.	149	99.3	50	100	199	
	HPV 56	Pos.	0	0	0	0	0	
		Neg.	150	100	50	100	200	
	HPV 58	Pos.	6	4	1	2	7	0.505
		Neg.	144	96	49	98	193	
	HPV 66	Pos.	0	0	0	0	0	
		Neg.	150	100	50	100	200	

Table (4.6): Association between age group and type of cervical cancer

		Age group						Total
		21-30	31-40	41-50	51-60	61-70	71-80	
Diagnosis	CIN 1	2	2	8	3	0	0	15
	CIN 2	1	3	4	3	2	0	13
	CIN 3	1	5	6	3	0	0	16
	SCC	5	16	35	15	8	1	80
	AdenoCa	1	2	7	5	4	0	19
	Adeno S.Ca	0	1	2	0	0	0	3
	Clear C.Ca	0	0	2	0	0	0	2
	Glassy C.Ca	0	0	2	0	0	0	2
	No C.Lesion	3	10	22	10	5	0	50
Total		13	39	88	40	19	1	200
P. Value		0.997						

Table (4.7): Association between age group and regions

		Age group						Total
		21-30	31-40	41-50	51-60	61-70	71-80	
Region	Azaal	5	5	12	10	7	1	40
	Aden	2	12	19	11	1	0	45
	Hadhramout	2	7	14	4	3	0	30
	Al-Janad	1	13	24	9	5	0	52
	Tohama	2	1	13	6	2	0	24
	Saba’a	1	1	6	0	1	0	8
Total		13	39	88	40	19	1	200
P. Value		0.320						

Table (4.8a): Association between age group and HR- HPV types

	HPV 16		HPV 31		HPV 33		HPV 35	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
21 – 30	8	5	0	13	1	12	0	13
31 – 40	20	19	3	36	2	37	0	39
41 – 50	35	53	5	83	2	86	2	86
51 – 60	9	31	1	39	1	39	2	38
61 – 70	2	17	1	18	0	19	2	17
71 – 80	0	1	0	1	0	1	0	1
Total	74	126	10	190	6	194	6	194
P.Value	0.005		0.856		0.791		0.302	

Table (4.8b): Association between age group and HR- HPV types

	HPV 18		HPV 39		HPV 45		HPV 59	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
21 - 30	3	10	0	13	1	12	0	13
31 - 40	8	31	2	37	2	37	1	38
41 - 50	12	76	1	87	4	84	1	87
51 - 60	7	33	1	39	1	39	2	38
61 - 70	2	17	1	18	2	17	0	19
71 - 80	0	1	0	1	0	1	0	1
Total	32	168	5	195	10	190	4	196
P. Value	0.832		0.741		0.844		0.714	

Table (4.8c): Association between age group and HR- HPV types

	HPV 52		HPV 56		HPV 58		HPV 66	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
21 - 30	0	13	0	13	1	12	0	13
31 - 40	1	38	0	39	1	38	0	39
41 - 50	0	88	0	88	2	86	0	88
51 - 60	0	40	0	40	1	39	0	40
61 - 70	0	19	0	19	1	18	0	19
71 - 80	0	1	0	1	1	0	0	1
Total	1	199	0	200	7	1953	0	200
P. Value	0.528				0.000			

Table (4.9a): Association between HR- HPV types and type of cervical cancer

	HPV 16		HPV 31		HPV 33		HPV 35	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CIN 1	2	13	0	15	1	14	1	14
CIN 2	5	8	3	10	1	12	0	13
CIN 3	11	5	3	13	0	16	1	15
SCC	46	34	2	78	3	77	2	78
Adeno.Ca	5	14	1	18	0	19	0	19
Adeno S.Ca	1	2	0	3	0	3	0	3
Clear C.Ca	1	1	0	2	0	2	0	2
Glassy C.Ca	1	1	0	2	0	2	0	2
No C. Lesions	2	48	1	49	1	49	1	49
Total	74	126	10	190	6	194	6	194
P. Value	0.000		0.018		0.914		0.140	

Table (4.9b): Association between HR- HPV types and type of cervical cancer

	HPV 18		HPV 39		HPV 45		HPV 59	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CIN 1	1	14	0	15	0	15	1	14
CIN 2	3	10	1	12	1	12	0	13
CIN 3	4	12	0	16	2	14	2	14
SCC	9	71	4	76	5	75	1	79
Adeno.Ca	12	7	0	19	0	19	0	19
Adeno.S.Ca	1	2	0	3	0	3	0	3
Clear C. Ca	1	1	0	2	0	2	0	2
Glassy C.Ca	0	2	0	2	0	2	0	2
No C. Lesions	1	49	0	50	1	49	0	50
Total	32	168	5	195	10	190	4	196
P. Value	0.000		0.621		0.813		0.122	

Table (4.9c): Association between HR- HPV types and type of cervical cancer

	HPV 52		HPV 56		HPV 58		HPV 66	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CIN 1	0	15	0	15	0	15	0	15
CIN 2	0	13	0	13	2	11	0	13
CIN 3	0	16	0	16	0	16	0	16
SCC	0	80	0	80	4	76	0	80
Adeno.Ca	0	19	0	19	0	19	0	19
Adeno.S.Ca	1	2	0	3	0	3	0	3
Clear C. Ca	0	2	0	2	0	2	0	2
Glassy C.Ca	0	2	0	2	0	2	0	2
No C. Lesion	0	50	0	50	1	49	0	50
Total	1	199	0	200	7	193	0	200
P. Value	0.000				0.398			

Table (4.10a): Association between HR-HPV types and region

	HPV 16		HPV 31		HPV 33		HPV 35	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Azaal	14	26	2	38	1	39	2	38
Aden	22	23	2	43	0	45	1	44
Hadhramout	7	23	1	29	1	29	0	30
Al-Janad	18	34	4	48	1	51	2	50
Tohamh	10	14	0	24	2	22	0	24
Saba'a	3	6	1	8	1	8	1	8
Total	74	126	10	190	6	194	6	194
P.Value	0.347		0.705		0.304		0.483	

Table (4.10b): Association between HR-HPV types and region

	HPV 18		HPV 39		HPV 45		HPV 59	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Azaal	9	31	1	39	3	37	0	40
Aden	4	41	2	43	3	42	3	42
Hadhramout	4	26	0	30	1	19	1	29
Al-Janad	10	42	2	50	3	49	0	52
Tohamh	3	21	0	24	0	24	0	24
Saba'a	2	7	0	9	0	9	0	9
Total	32	168	5	195	10	190	4	196
P.Value	0.551		0.746		0.736		0.166	

Table (4.10c): Association between HR-HPV types and region

	HPV 52		HPV 56		HPV 58		HPV 66	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Azaal	0	40	0	39	1	39	0	39
Aden	0	45	0	45	0	45	0	45
Hadhramout	0	30	0	27	3	27	0	27
Al-Janad	1	51	0	52	2	50	0	52
Tohamh	0	24	0	27	0	24	0	27
Saba'a	0	9	0	10	0	9	0	10
Total	1	199	0	200	7	193	0	200
P.Value	0.721		-		0.380		-	

Table (4.11): Association between regions and type of cervical cancer

	Azaal	Aden	Hadhramout	Al-Janad	Tohama	Saba'a	Total
CIN 1	3	1	3	4	3	1	15
CIN 2	3	3	5	2	0	0	13
CIN 3	2	5	1	5	2	1	16
SCC	15	21	8	21	11	4	80
Adeno Ca	6	2	3	3	4	1	19
Adeno S.Ca	0	0	0	2	0	1	3
Clear Cell Ca	0	0	0	2	0	0	2
Glassy Cell Ca	0	2	0	0	0	0	2
No Cervical Lesion	11	11	10	13	4	1	50
Total	40	45	30	52	24	9	200
P. Value	0.319						

Table (4.12): Association between immunohistochemistry results and age group

		Age Group						Total
		21 - 30	31 - 40	41 - 50	51 - 60	61 - 70	71 - 80	
Immuno Results	Pos.	8	23	35	13	5	0	84
	Neg.	5	16	53	27	14	1	116
Total		13	39	88	40	19	1	200
P. Value		0.052						

Table (4.13): Aassociation between immunohistochemistry results and region

		Region						Total
		Azaal	Aden	Hadhramout	Al-Janad	Tohama	Saba'a	
Immuno Results	Pos.	17	19	10	23	12	3	84
	Neg.	23	26	20	29	12	6	116
Total		40	45	30	52	24	9	200
P. Value		0.857						

Table (4.14): Association between immunohistochemistry results and type of cervical cancer.

		Immuno Results		Total
		Pos.	Neg.	
Diagnosis	CIN 1	4	11	15
	CIN 2	7	6	13
	CIN 3	10	6	16
	SCC	45	35	80
	AdenoCa	8	11	19
	Adeno Squamous Ca	3	0	4
	Clear Cell Ca	1	1	2
	Glassy Cell Ca	0	2	2
	Non C.Lession	6	44	50
Total		84	116	200
P. Value		0.000		

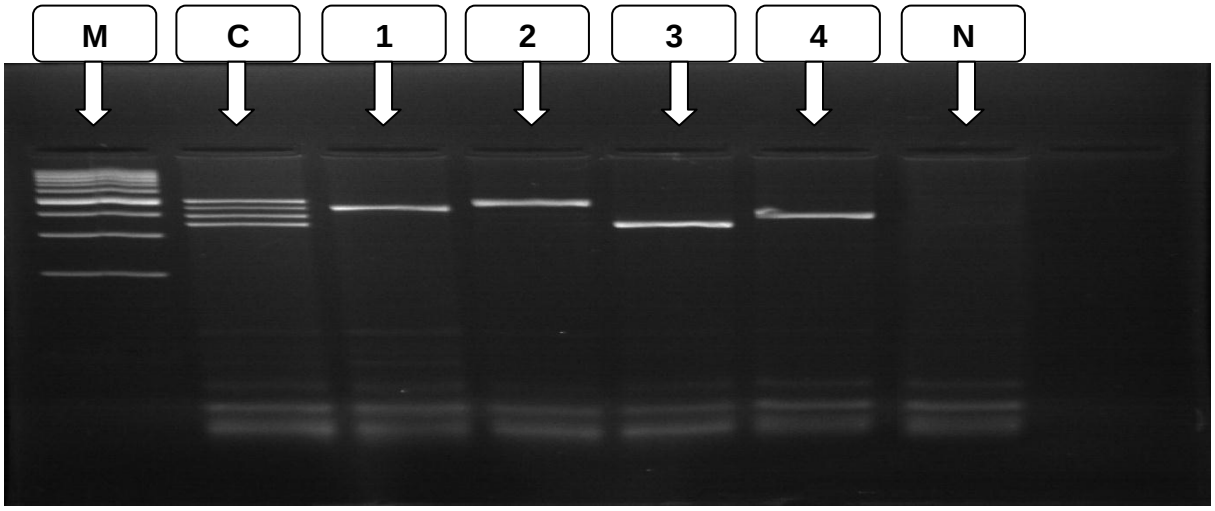


Figure (4.2): PCR amplification of high risk HPV in cervical lesions samples. Lane M: marker (1000.bp DNA ladder), Lane C positive control for HPV 16, 31, 33, and 35, lane 1, 2, 3, 4 positive samples, lane 1 positive for HPV 16, lane 2 positive for HPV 31, lane 3 positive for HPV 33, lane 4 positive for HPV 35, lane N negative control.

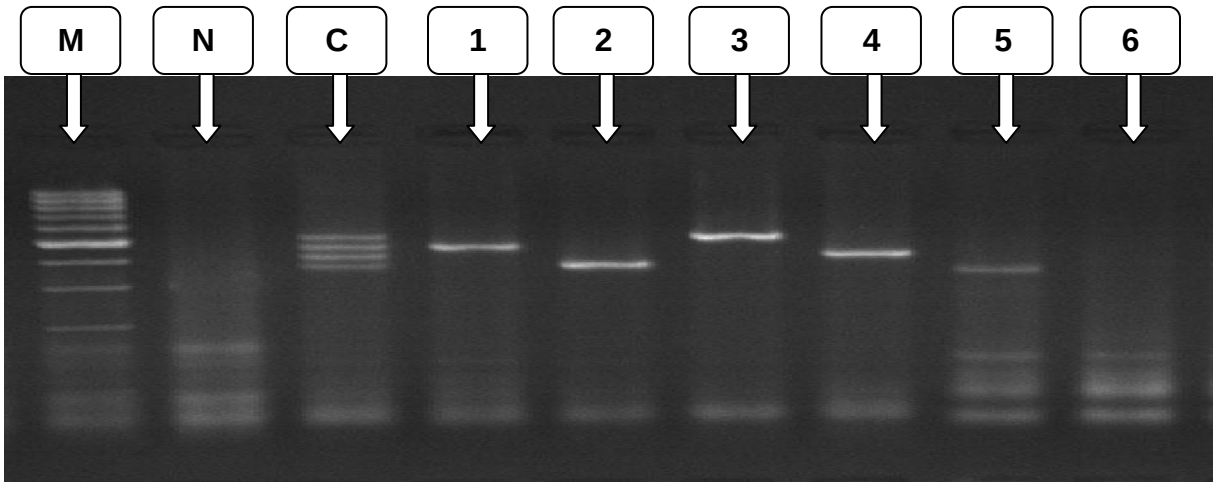


Figure (4.3): PCR amplification of high risk HPV in cervical lesions samples. Lane M: marker (1000.bp DNA ladder), lane N negative control, lane C positive control for HPV 18, 39, 45, and 59, lane 1 positive for HPV 18, lane 2 positive for HPV 39, lane 3 positive for HPV 45, lane 4 positive for HPV 59, lane 5 positive for HPV 39, lane 6 negative sample.

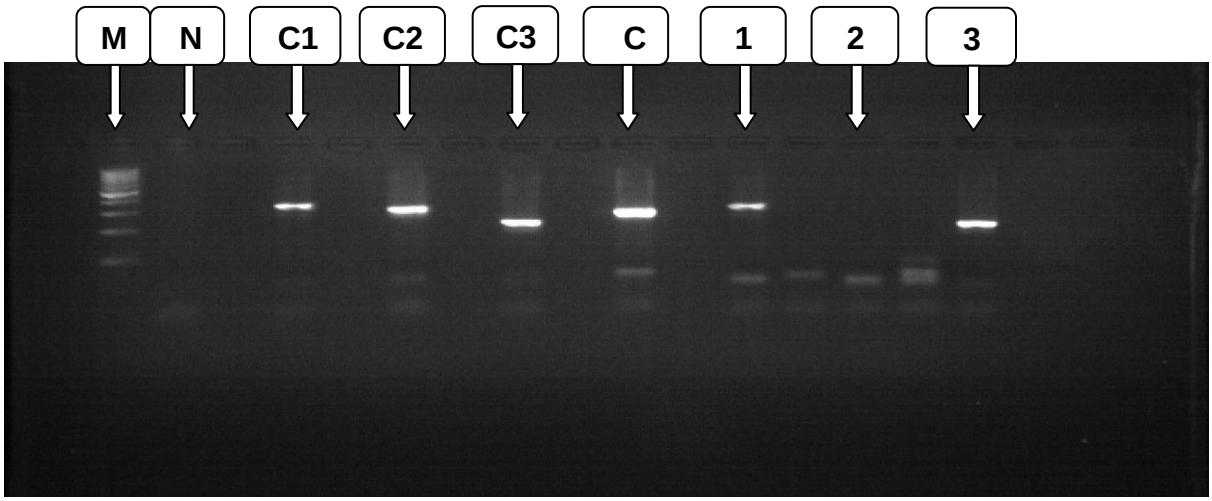
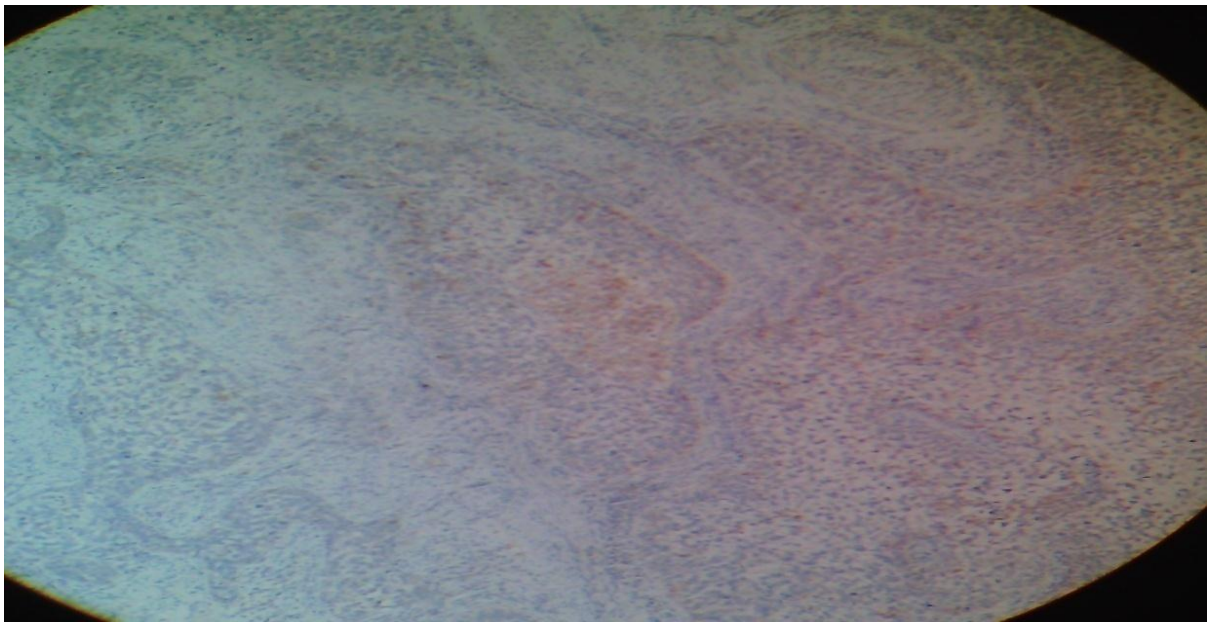
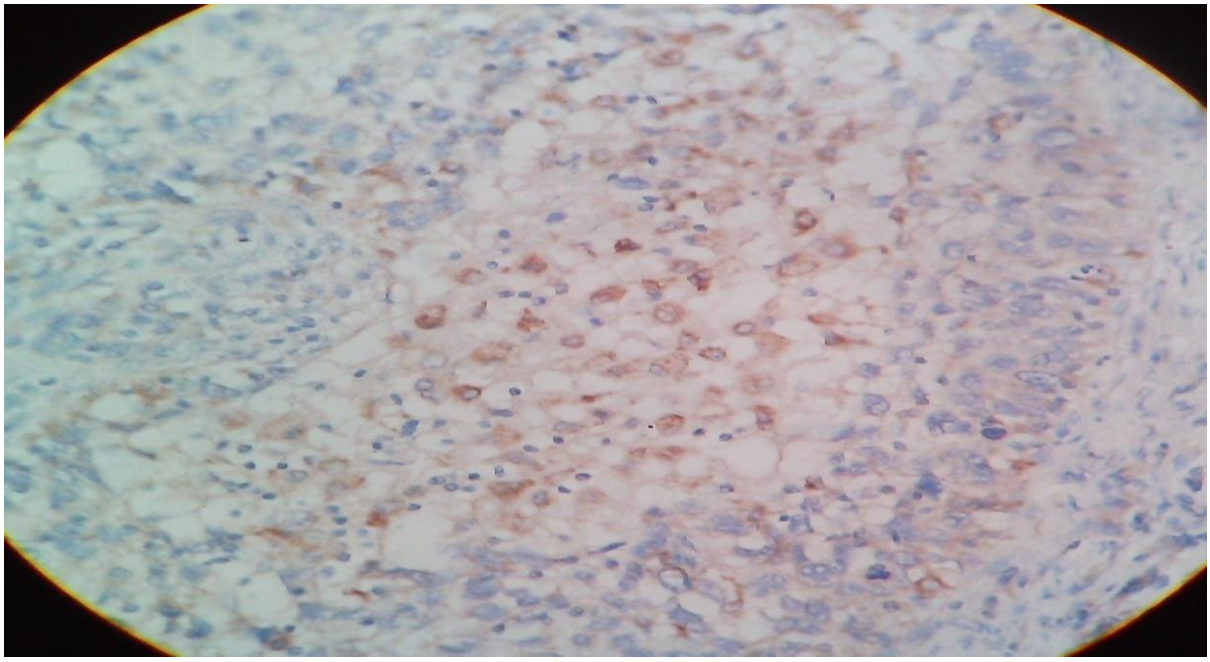


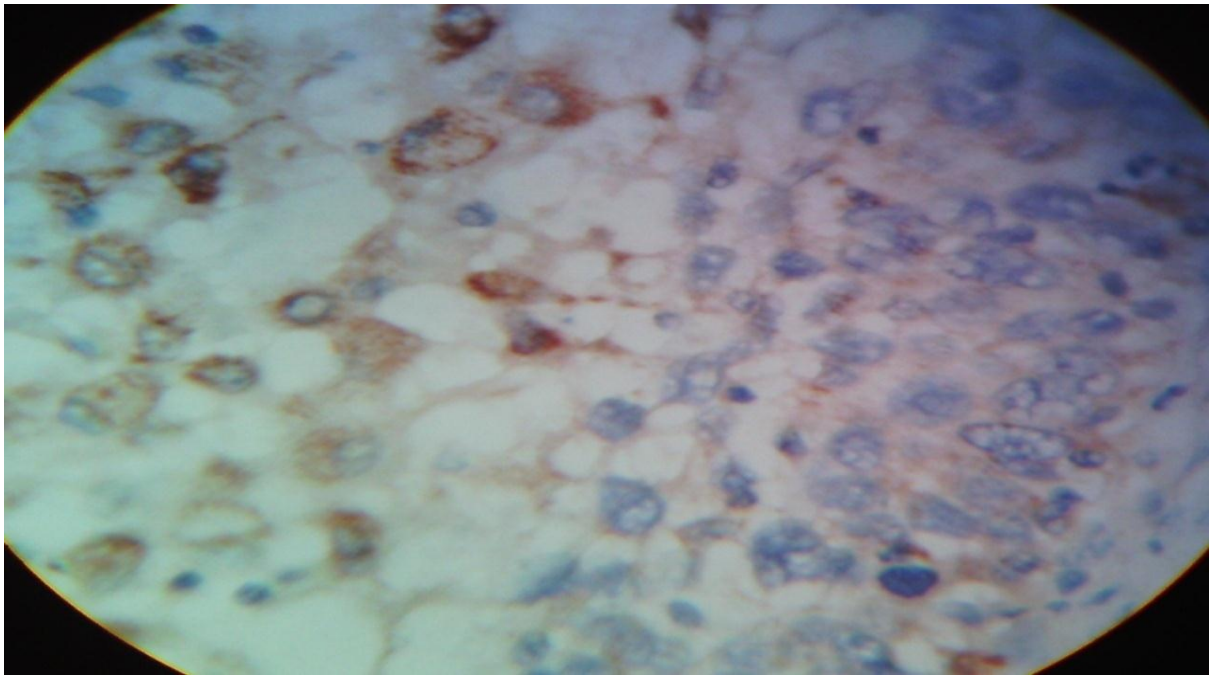
Figure (4.4): PCR amplification of high risk HPV in cervical lesions samples. Lane M: marker (1000.bp DNA ladder), lane N negative control, lane C1 positive control for HPV 52, lane C2 positive for HPV 56, lane C3 positive for HPV 58, lane C4 positive for HPV 66, lane 1 positive for HPV 52, lane 2 negative sample, lane 3 positive for HPV 58.



Photomicrograph (4.1): Cervical cancer tissue (10X) K1H8 (anti-HPV) Immunohistichemical staining: positive.



Photomicrograph (4.2): Cervical cancer tissue (40X) K1H8 (anti-HPV) Immunohistichemical staining: positive.



Photomicrograph (4.3): Cervical cancer tissue (100X) K1H8 (anti-HPV) Immunohistichemical staining: positive.

Chapter five

Discussion

Cervical cancer is the second most common cancer affecting women worldwide (Mohamed, *et al*. 2012; Abdelbaset, *et al*. 2012; Qmichou, *et al*. 2013). It is now generally agreed that persistent infection by high-risk HPV genotypes is the major cause of cervical cancer (Nyasha, *et al*. 2014). Many studies have been conducted to determine the most frequent HPV genotypes in different parts of the world, but there is no such study in Yemen. To the best of our knowledge this is the first study to determine the most frequent HPV genotypes in Yemen. Data is not yet available on the HPV burden in the general population of Yemen (Castellsague, *et al.* 2007). However, in Western Asia, the region to which Yemen belongs, about 2.2% of women in the general population are estimated to harbor cervical HPV infection. Moreover, about 66.7% of invasive cervical cancers are attributed to HPV subtypes 16 or 18. Incidence of cervical cancer in Yemen has been reported to be 1.4 per 100,000 women in 2008 (Maha Abdul-Aziz, 2012).

In the present study SCC and CIN 1, 2, 3 were commonly diagnosed representing together 82.6% of the total cases of cervical cancer. Similar findings were reported from other parts of the world; squamous cell carcinoma was reported to be the most common histologic subtype of cervical cancer (Levison, *et al.* 2008; Stevens, *et al*. 2009; Howlader, *et al.* 2013). Asimilar studies by Schneider, *et al*. (2009) and Kumar, *et al*. (2010) reported that the SCC accounted for approximately 80% of cervical cancer cases.

The current study has provided evidence of strong association between high-risk HPV types and risk of cervical cancers (P <0.000); the prevalence of HR-HPV

was 76% among all cancer cases. These findings seen to be agree with previous studies worldwide (Kawana, *et al*. 2012, Sitakan, *et al*. 2013; Nyasha, *et al*. 2014). This study also reported that the HPV 16 and 18 were the most common HPV types among Yemeni women which were detected in 68.7% of the cancer cases followed by HPV 45 and 31. These results agree with several studies worldwide; Cosette, et al. (2009) found that the most common HPV genotypes detected in invasive cancers were HPV16 (53.2%), HPV18 (13.1%), and HPV45 (6.1%) and those in in-situ cancers were HPV16 (56.3%), HPV31 (12.6%), and HPV33 (8.0%). A similar study reported that the five most common HPV types identified as single types among HPV-positive cases were HPV16 (64.7%), HPV18 (9.9%), HPV45 (9.9%), HPV31 (3.0%), and HPV33 (2.2%). The study showed that in Turkey, HPV16/HPV18 accounted for 75.4% of HPV-positive ICC cases (Usubutun, *et al.* 2009). A study conducted in China by many researchers; they revealed that, HPV16 was the most prevalent type in lesions in both urban and rural settings. Combined, HPV16 and 18 were constituted 71.4% of HPV-positive CIN2+ lesions (Zhang, *et al*. 2013). Pirog, et al. (2014) found that three HPV genotypes, HPV 16, 18, and 45, dominated in all cervical adenocarcinomas and together accounted for 94.1% of HPV-positive tumors. HPV16 was the most common and found in 50.9% of HPV-positive cases, followed by HPV18 (31.6%) and HPV45 (11.6%). In Iran 2011, many researchers found that the overall genotyping results of phylogenetic analysis and hybridization methods were as follows: HPV 16: 75% (65/87); HPV 18: 3% (2/87); HPV 31: 1% (1/87); HPV 45: 1% (1/87) (Shahsiah, *et al*. 2011). Other studies with relatively similar findings as in Brazil Cristina, *et al.* (2013) found that 170 (99%) out of 172 valid ICC samples were HPV DNA positive. The most frequent types were HPV16 (77.6%), HPV18 (12.3%),

HPV31 (8.8%), HPV33 (7.1%) and HPV35 (5.9%). Most infections (75%) were caused by individual HPV types. Also in Northeast Brazil Fernandes, et al. (2013) found that overall HPV prevalence was 65.2% (277/425), with 85.9% (238/277) single and 14.1% (39/277) multiple infection. The most prevalent HPV types were HPVs 16, 58, 18, 31, and 45. In Egypt Abd El-Azim, et al. (2011) found that in patients with invasive CCA, 93.3% (28/30) were positive for HPV DNA. In order of decreasing frequency, HPV genotypes were: HPV 16 being detected in 66.7% (20/30), HPV 18 in 16.7% (5/30), HPV 33 in 10.0% (3 /30) and both HPV 31 and HPV 45 in 6.7% (2/30) cases. In sub-Saharan Africa many researchers found that the most commonly detected HPV types were HPV16 (51.2%), HPV18 (17.2%), HPV35 (8.7%), HPV45 (7.4%), HPV33 (4.0%) and HPV52 (2.2%) (Denny, *et al*. 2014). Also Quek, *et al*. (2013) across 5 countries in Asia, namely, Malaysia, Vietnam, Singapore, South Korea, and the Philippines, found that the most common types of HPV observed among ICC cases were HPV 16 (36.8%-61.3%), HPV 18 (12.9%-35.4%), HPV 52 (5.4%-10.3%), and HPV 45 (1.5%-17.2%), whereas among CIN 2/3 and AIS cases, HPV 16 (29.7%-46.6%) was the most commonly observed type followed by HPV 52 (17.0%-66.7%) and HPV 58 (8.6%-16.0%).

Mohammadreza, *et al*. (2013) found that of the 98 cervical samples analyzed by DNA PCR, 78 (79.59%) were positive for HPV DNA. HPV was detected in the 52 of SCC, 4 of Adenocarcinomas, 14 of CIN-I, 4 of CIN-II, and 4 of CIN-III. From the 78 HPV positive cervical samples, 23 (29.5%) samples were positive for HPV type 16, 32 (41%) for HPV 18, 19 (24.4%) for HPV 45, and 4 (5.1%) were positive for HPV 39. In Saudi Arabia Alsbeih, *et al*. (2011) found that eighty-nine of the ICC specimens were HPV-positive. Eleven different HPV genotypes were detected, 8 high risks (16, 18,

31, 39, 45, 51, 59, and 73) and 3 low risk (6, 64, and 70). Ten patients had double infections involving mainly HPV-16 and 18. The most common genotypes were 16 (65.2%), 31 (7.9%), 45 (6.7%), 18 (3.4%), and 73 (2.3%). In contrast Mbaye, *et al*. (2014) found that the prevalence of HR-HPV infections in Dakar Region (Senegal) was 17.4%. HPV 52 (3.2%) was the most prevalent type, followed by HPV 31 (3.0%) and HPV 16, 45, and 53 (all 2.8%). This little variation of our findings with that other investigations is largely explained by differences in the age range of the populations studied and the sensitivity of the DNA assay used for detection of HPV infection also it might be due to geographic variation, sample storage time and various etiological risk factors.

Moreover in the present study we found that the HPV 16 was most common among SCC and CIN3 samples while, HPV 18 was more common among adenocarcinoma. These findings are in agreement with the study reported by Prithwijit, *et al*. (2014), in which HPV was detected in 97.5% (39/40) of cases. HPV16-infected cases (32/39; 82.05%) predominated over HPV18-infected ones (7/39; 17.95%). However, HPV18 single infection was significantly more among adenocarcinoma (3/4; 75%) than SCC (2/26; 7.69%) contrary to HPV16 single infection (SCC = 24/26, 92.31%; adenocarcinoma = 1/4; 25%) whereas both CIN3 cases were HPV16-positive. This study also noted that HPV 31 was detected mainly as co-infection with other HPV types, this finding gives support to a study conducted in China which reported that, HPV31 always occurred as a co-infection with another HPV type and therefore was attributed minimal causality (Zhang, *et al*. 2013). This study also showed that the HPV 16 is more common in the age groups less than 50 years. Several other studies revealed relatively similar findings. In Kuwait 69% of all

HPVs were found in women aged 20-29 years, and the HPV incidence rate deceased with increasing age (Al-Awadhi, *et al*. 2013). Another study reported that, invasive cancer case subjects who were positive for HPV16 or 18 were diagnosed at younger ages than those who were positive for other carcinogenic HPV genotypes (Cosette, *et al*. 2009). A similar study showed that the patients' median age was significantly lower (P=0.028) in HPV-16/18 infected groups compared to other genotypes (Alsbeih, *et al*. 2011). In contrast another study reported that the patients with HPV-18 infection had a younger age (P=0.009) and higher tumor grade (P<0.001) than patients with HPV-16 infection (Siriaunkgul, *et al*. 2013). These differences with the present studt may be due to unequal number of patients within each age group as 44% of the study population age ranged from 41-50 years and 6.7% were in the age group 21-30 years while about 10% were more than 60 years old; other risk factors could be involved. In this study also we found no significant association btween cervical cancer types and HPV prevalence in the different geographical regions. Again this may be due to unequal distribution of the study population among the regions investigated or due to exposure to simillar risk factors. There are many risk factors that may be play a major role in the development of cervical cancer in Yemen, like multi-parity, early age of marriage, use of oral steroid contraceptives, cigarette smoking, some dietary factors, lack of knowledge about screening of cervical cancer and its prevention and socioeconomic factors.

In this study we also observed that the PCR was more sensitive than immunohistochemistry techniques in the detection of HR-HPV; by PCR 114/150 (76%) of cases were reported positive for HPV, while by immunohistochemically only 78/150 (52%) of cases were positive for HPV. Many studies reported that the most

perspective way of HPV diagnosis is a direct detection of DNA of the human papilloma virus of high carcinogenic risk by the polymerase chain reaction (Kosel, *et al.* 2003; Hopman, *et al.* 2005; Giovanna, *et al.* 2012). It has also been reported that PCR is the most powerful tool for the epidemiological investigation of HPV infection or cervical cancer, and it is the gold standard test for HPV research in which the natural history of HPV infection has been more clearly defined with PCR (Coutlee, *et al*. 1997). So, PCR is better than immunohistochemistry in the detection of HPV. By PCR we can do genotyping of HPV and also detect multiple infections that can not be revealed by immunohistochemistry.

There are clear limitations in our material when investigating the prevalence of HPV. The patients with cervical lesions were selected and samples were not processed at the same time as the normal cervical and tumor samples. Also we do not have enough knowledge about the patients, like parity, age of marriage, use of oral steroid contraceptives, cigarette smoking, patient's socioeconomic status, nutritional status, education, previous health history nor family relations. In summary, the patient data reinforce the clinical importance of HPV-associated cervical cancer in Yemeni population. The high prevalence of HPV 16, 18, 45, 31 and 58 genotypes suggests towards vaccination for HPV genotypes as an important parameter for reducing cancer risk due to HPV infection.

Conclusions and Recommendations

Conclusions:

On the bases of this study we conclude the following:

1- The most prevalent cervical cancer in Yemeni women is SCC in which together with CIN1, 2, 3 represent about 82% followed by adenocarcinoma.

2-There is a strong association between HR-HPV and cervical cancer with prevalence rate of 76% among cases.

3- HR-HPV infections proved to be the most important factor that leads to cervical pathological changes among Yemeni women that progress to cervical cancer.

4- HPV 16 is the most commonly detected genotype in positive cases often in association with SCC in women under 50 years of age.

5- HPV 18 is the second commonly detected genotype always associated with adenocarcinoma.

6- HPV types 16, 18, 45, 31 and 58 are the most common types in Yemeni patients.

7- Multiple HPV infections are fairly common (27% of positive cases) mainly involving HPV16, 18 and 31.

8- PCR is more sensitive than immunohistochemistry in detecting single and multiple infections of HPV.

Recommendations:

On the bases of this study we recommended that:

1- public awareness about cervical cancer and associated risk factors should be raised through regular health education programs.

2- Health Screening Programs for detection of cervical cancer and HPV is very important and should be initiated.

3- Females younger than 12 years old be vaccinated against HR-HPV

4- Further comprehensive studies should be carried out using larger sample size considering all risk factors and covering more regions.

5- Immunohistochemical method can be used alone as a detection technique for HPV in cervical cancer but it is better to combined with molecular methods.

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Appendix I

Materials and instruments used for preparation processing and staining of the specimen include:

Instruments:

- Polymerase chain reaction (PCR) machaine
- Water bath
- Rotary microtome
- Desktop microcentrifuge for eppendrof type tubes
- Gel documentation system (Gel mega, digital camera and software in a computer).
- Pipettors (capacity 0,5-10 µl; 5-40µl; 40-200µ, 200-1000µ)
- Freezer
- Refrigerator
- Silanized coated slides
- Eppendorf tube
- Disposable gloves.
- Forested end glass slides (75×25×2 mm).
- Cover glass.
- Disposable microtome blade
- Vortex mixer
- Tube racks
- Pencil.
- Permanent Marker.
- Cytomension pen
- Humidity chamber

Materials:

A- for immunohistochemistry:

- Mayer´s haematoxylin
- Ethyl alcohol.
- Distilled water (D.W).
- DPX.
- Xylene
- Target Retrieval Solution

- Antibody diluent
- Monoclonal mouse anti-human papillomavirus clone K1H8
- ICH wash buffer (PBS)
- 3% hydrogen peroxide
- Biotinylated link
- Streptavidin-HRP
- DAB+ substrate buffer
- DAB+ chromogen

B- for molecular biology (PCR)

- Xylene
- Distilled water (D.W).
- Tris-Borate EDTA buffer (TBE)
- Ethedium bromide
- Lysis solution
- Washing solution 1
- Washing solution 2
- Sorbent
- DNA-eluent
- Ethanol 100%
- **PCR-mix-1 "16-35"**(primers directed against regions of *HPV 16,31,33,35),*0,275 mL
- **PCR-mix-1 "18-59"**(primers directed against regions of *HPV 18,39,45,59*),0,275 mL
- **PCR-mix-1 "52-66"**(primers directed against regions of *HPV 52,56,58,66*),0,275 mL
- 2,5 x buffer- 3 x 0,6 mL
- TaqF Polymerase, 0,09 mL
- Mineral Oil, 8,0 ml
- DNA-buffer (C-), 0,5 mL
- **HPV Genotype Controls Panel** (types 16, 31, 33, 35; 18, 45, 39, 59; 52, 56, 58, 66), 12 x 0,15 ml.

C- Preparation:

- Mayer´s haematoxylin components:

Haematoxylin powder…….…… 1 g.

Potassium or ammonuium alum ……..…..…… 50 g.

Sodium iodate ……..……..…..……... 0.2 g.

Citric acid …….…….…..... 40 ml

Chloral hydrate ……..…..…… 50 g

Distilled water ……..…… 1000 ml.

Eosin preparation:

Eosin powder ……..……. 1g

Distilled water ……..…… 100 ml.

Tris-Borate EDTA buffer (TBE) component:

Stock concentration 5.0X and working concentration 0.5X

The stock concentrations 5.0X:

Tris Base ……..…….. 54 g

Boric acid ……..……. 27.5 g

EDTA ……..…… 20 ml

Distilled water ……... up to 1 liter.

Ethedium bromide:

Stock concentration (in water) 10 mg/ml

Bromide powder ……..…….. 0.1g

D W …….. 10 ml

Working concentration (in TBE agarose) 0.5 µg/ml

Appendix II

Information about Yemen:

Location:

Yemen is located in the Middle East at the southern tip of the Arabian Peninsula, bordering the Arabian Sea, Gulf of Aden, and the Red Sea. It lies south of Saudi Arabia and west of Oman, between latitudes 12° and 19° N and longitudes 42° and 55° E. It is situated at the entrance to the Bab el Mandeb strait, which links the Red Sea to the Indian Ocean (via the Gulf of Aden) and is one of the most active and strategic shipping lanes in the world.

Size:

Yemen has an area of 527,970 square kilometers, including the islands of Perim at the southern end of the Red Sea and Socotra at the entrance to the Gulf of Aden.

Major Cities:

The capital of Yemen is Sanaa. Other major cities are Aden, Taizz, Al Hudaydah, and Al Mukalla (LC-FRDCP, 2008)

Population:

The population of Yemen is 26,052,966 (July 2014 est.) with 46% of the population being under 15 years old and 2.7% above 65 years. In 1950, it was 4.3 million. By 2050, the population is estimated to increase to about 60 million. Yemen has a high total fertility rate, 4.09 children born/woman (2014 est.) It is the 30th highest in the world (CIA, 2014).

Regions:

Under the new system, Yemen is divided into six regions that include four in the north, comprising Azal, Saba, Al-Janad and Tahama, and two in the south, Aden and Hadramawt.

Azal includes the capital Sanaa, in addition to the provinces of Dhamar, Amran and Saada, Aden comprise the capital of the former south, as well as Abyan, Lahej and Daleh. The southeastern Hadramawt province include Al-Mahra, Shebwa and the island of Socotra, while Saba comprises Bayda, Marib, Al-Jawf and Dhamar. Al-Janad would include Taez and Ibb, and Tahama also takes in Hudaydah, Rima, Mahwit and Hajja (YNDC, 2014).

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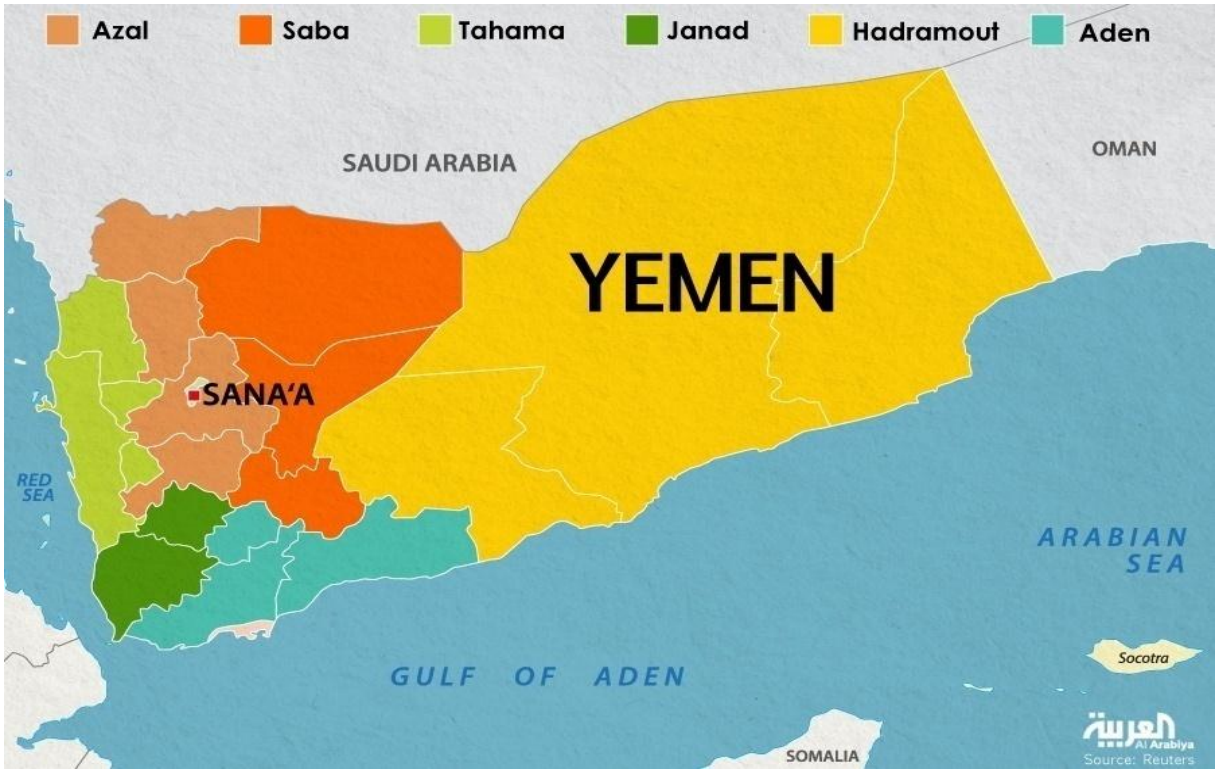

Figure: Maps of Yemen (Yemen's new regions).