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Immunohistochemical Study and in-situ hybridization for detection of humanpapillomavirus (HPV) in Uterine Cervical Carcinoma

A thesis

Submitted To The College Of Medicine And the Committee Of Postgraduate Studies Of Kufa University In Partial Fulfillment Of The Requirements For The Degree Of Master Of Science In Pathology (Histopathology)

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بِسْمِ اللهِ الرَّحْمـَنِ الرَّحِيمِ

نَرْفَعُ دَرَجَاتٍ مِّن نَّشَاء وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ

صَدَقَ اللّهُ الْعَلِيُّ الْعَظِيمُ

سورة يوسف , الآية (76)

Dedication

To …

My Family

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bp base pair

CDK/cdk	cycline dependent kinase
CIN	Cervical intraepithelial neoplasia
CIP-1	Cdk inhibiting protein 1
CIS	Cervical carcinoma in situ
CMI	Cell-mediated immune responses
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DPX	Distyrene, Plasticizer and Xylene
FDA	The Food and Drug Administration
FFPE	Formalin – fixed, paraffin - embedded tissue specimens
FIGO	Federation International of Gynecology and Obstetrics Staging System
FISH	Filter in Situ hybridization
G0	cell cycle stationary phase
G1	is the phase in which the cell gears up for division
G2	is the space before mitosis
H & E	Haematoxylin and Eosin
HCII	Hybrid Capture II (commercially available HPV test)
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human Papillomavirus
HR-HPV	High-Risk HPV type

HRP	horseradish peroxidase
HSIL	High-grade squamous intraepithelial lesions
HSV	Herpes simplex virus
Ig	Immunoglobulin
IgA	Immunoglobulin class A
IgG	Immunoglobulin class G
IHC	Immunohistochemistry
INK4	inhibitor of cdk4/6
ISCC	Invasive squamous cell carcinoma
ISH	In Situ hybridization
KIP	Kinase inhibitory protein
LCR	Long control region
M	cell cycle mitotic division phase
MHC	Major Histocompatibility complex
NK	Natural killer
OCs	Oral contraceptives
ORF	Open reading frame
P	Protein
Pap	Papanicolaou smear
PAP	Peroxidase-anti-peroxidase reaction
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

Rb	Retinoblastoma protein
RNA	Ribonucleic acid
SILs	Squamous intraepithelial lesions
S-phase	cell cycle phase in which new nucleic acid is made
SPSS	SPSS Statistical Package for the Social Science
STD	Sexual transmitted disease
TBS	Tris-buffered saline
TNM	Staging System (T: tumor, N: lymph node, M: metastasis)
TSA	Tyramide Signal Amplification
URR	Upstream regulatory region
VIA	Visual inspection of the cervix after application of acetic acid
VILI	Visual inspection of the cervix after application of Lugol's Iodine
VLP	Virus-like particle
Vs	Versus
WHO	World Health Organization

Summary

Background

Cervical cancer is the second most common life-threatening cancer among women worldwide. The human papilloma virus (HPV) has been definitely linked as the main etiologic agent for cervical carcinoma. To date, there are more than 200 known HPV types, and they are generally classified according to their potential to induce malignant transformation. HPVs 16,18, 31, 33, 35, 39, 45, 50, 51,53, 55, 56, 58, 59, 64 and 68 are considered “high risk” types because they are detectable in carcinomas and dysplasias and low risk types (non-oncogenic:6,11,40,42,43,44,54,61,72,73,81). Immunohistochemistry was

performed for detection of HPV proteins on cervical tissues. Chromogenic in situ hybridization was performed to detect HPV DNA and determine high-risk HPV on cervical samples.

Aim of the Study

This study was designed to assess rate of detection and prevalence of HPV in cervical carcinoma hysterectomized patients through both immunohistological and chromogenic in-situ hybridization techniques and to identify and to differentiate histopathologically the high risk genotype of HPV (16, 18) from low risk genotype (6,11).

Materials and Methods

A total 75 cervical tissue samples of female patients, who had undergone hysterectomy or submitted for punch biopsy from their cervices, were included in this study, that conducted in the Department of Pathology, Faculty of Medicine, Kufa University from December 2010 through June 2011. Forty three patients with cervical carcinoma (57.3%) (40 patients with squamous cell carcinoma and 3 with adenocarcinoma) and the remaining 32(42.7%) normal cervical tissue were considered as control group involved in this study. All the cases were collected from Al-Sadder Teaching Hospital and some of the private laboratories in Najaf. Their ages were ranging between 28-65 years with a mean age of 46.02 years. Labeled Streptavidin -Biotin ($LSAB^{+}$) method and GenPoint ^{TM} Tyramide Signal Amplification System were employed for immunohistochemical and chromogenic in situ hybridization detection of HPV respectively. Statistical analysis was achieved by Chi-square test and correlation-regression test at p value< 0.05 and ≥ 0.3 respectively.

Results

The immunohistochemistry detection rate of HPV 6/11/16/18/ 31/33/ 35/45/ 51/52 was reported in 18 (41.9%) out of 43 cases of cervical carcinoma. Chromogenic in situ hybridization detection rate of HPV 6/ 11/16/ 18/ 31/ 33/ 35/ 45/ 51/52 was reported in 15 (34.9%) out of 43 cases of cervical carcinoma .However Chromogenic in situ hybridization detection rate of high risk genotypes of HPV(16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was reported in 17 (39.5%) out of 43 cases of cervical carcinoma. All normal control and adenocarcinoma cases showed no specific signals for HPV DNA and protein . There was a significant difference of HPV immunoexpression among types of cervical tissue and age of the patients . However, HPV was positively correlated with tumor grade and size(T) of tumor. A significant correlation was found between both detection methods of HPVs (immunohistochemistry and CISH techniques) in the cases of cervical carcinoma.

Conclusions and recommendations

From the above results we concluded that HPV over-expression plays an important role in pathogenesis of cervical carcinoma . So incorporation of this marker with other pathological parameters will more accurately predict clinical outcome and determine the effects of anti cancer therapy.

Introduction and Review of literature

Introduction

Cervical carcinoma is the second leading cause of the death in women worldwide . It accounts for 10% of all cancer worldwide [(1)] . Cancer of the cervix uteri is the second most common cancer among women worldwide and ranks the tenth within the leading cancers for females in Iraq [(2)]. The Pap test is a screening tool that identifies women likely to have pre-malignant disease and high risk for cervical cancer. The incidence and mortality form invasive cervical cancer has fallen in communities where intensive screening has been carried out [(3)].

Over 80% of women newly diagnosed live in developing countries; most are diagnosed when they have advanced disease, and estimated 95% of women in developing countries have never been screened for cervical cancer [(4)].

In contrast ,the incidence of cervical carcinoma in countries of the region is low, as it is also the cases for Islamic countries. There are exceptions where the incidence is on the increase. Lack of cervical awareness by the population, providers and policy makers and lack of poor quality of screening programs result in cancer being diagnosed at later stages [(5)].

Modifying factors may either impact on neoplasia by directly influencing the processes underlying carcinogenesis, or indirectly by affecting persistence of viral infections. HPV is implicated as promoter for cervical carcinoma and certain strain of HPV associated with cervical cancer. They are capable of integrating the viral genome into host DNA forming a complex which abrogate the normal response of cervical epithelial cell cycle to DNA damage , which may allow to

inactivation of some tumor suppressor genes products that lead to progressive uncontrolled cell growth [6] .

HPV is member of a group of small double stranded DNA viruses which are casually associated with anogenital carcinoma and particularly with cervical carcinoma. The mechanisms by which the HPVs function in malignant progression appear predominantly to involve the activity of the viral oncoproteins [7] .

Most investigative works have pointed the virus as an oncogenic agent which implicates as promoter of cervical cancer . There are more than 200 subtypes of HPV ,including low risk types and high risk types [8] .

As the size of the problem and the incidence of HPV infection in Iraq are still underestimated, this work is an attempt to explore their influence and participation in the pathogenesis of cervical carcinoma in middle of Iraq.

Aim of the study

1- To assess rate of detection of HPV in both cervical carcinoma patients and hysterectomized patients presented for other causes.

2- To compare the efficiency of both immunohistological and CISH techniques in detection of HPV in uterine cervical biopsies .

3- To identify and differentiate histopathologically the high risk genotype of HPV (16, 18) from low risk genotype (6,11).

1.1.Cervical carcinoma

The cervix is the lower part of the uterus (womb) and connects the body of the uterus to the vagina (birth canal). The part of the cervix closest to the body of the uterus is called the endocervix. The part next to the vagina is the exocervix (or ectocervix). There are two main types of cells covering the cervix, squamous cells (on the ectocervix) and glandular cells (on the endocervix). The area between the original squamo-columnar junction and new squamo-columnar junction is called the transformation zone. Most cervical carcinomas start in the transformation zone [9,10].

Cervical carcinoma is a malignant neoplasm of the cervical epithelium of the uterus. It may present with vaginal bleeding, but symptoms may be absent until the cancer reached its advanced stages [11].

The normal epithelial cells of the cervix first gradually develop precancerous changes that turn into cancer. Although cervical cancers start from cells with pre-

cancerous changes (pre-cancers), only some women with pre-cancers of the cervix will develop cancer [12,13].

1.2.Epidemiology and Incidence of cervical cancer

Iraq has a population of 8.21 millions women of ages 15 years and older who are at the risk of developing cervical cancer. Current estimates indicate that every year 311 women are diagnosed with cervical cancer and 212 die from the disease. Cervical cancer ranks as the 10th most frequent cancer among women in Iraq, and the 7th 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 Iraq. However, in Western Asia where Iraq belongs to, about 2.2% of women in the general population are estimated to harbor cervical HPV infection at a given time[14](Table.1.1).

Table 1.1: Key Statistics on Iraq[14] .

Population

Women at risk for cervical cancer (Female population aged >=15 yrs) 8.21 millions	
Burden of cervical cancer and other HPV-related cancers	
Annual number of cervical cancer cases	**311**
Annual number of cervical cancer deaths	**212**
Projected number of new cervical cancer cases in 2025*	**568**
Projected number of cervical cancer deaths in 2025*	**393**

Projected burden in 2025 is estimated by applying current population forecasts for the country and assuming that current incidence/mortality rates of cervical cancer are constant over time. Rates per 100,000 women per year.

Cervical cancer is one of the most frequent diseases in women. It comprises approximately 12% of all cancers in women worldwide and the second most common type, after breast cancer, with an estimated 529,409 new cases and 274,883 deaths in 2008. About 86% of the cases occur in developing countries. Incidence of cervical cancer in Asia is lower, but varies significantly between sub-regions and also countries [(14)] .

This section describes the current burden of invasive cervical cancer in Iraq, Western Asia region and the world with estimates of annual number of new cases, deaths, and incidence and mortality rates(Table 1.2).

Table 1.2: Incidence of cervical cancer in Iraq, Western Asia and the World [14] .

Indicator	Iraq	Western Asia	World
Crude incidence rate	2.1	3.6	15.8
Age-standardized incidence rate	3.1	4.5	15.3
Cumulative risk (%). Ages 0-74 years	0.3	0.5	1.6
Annual number of new cancer cases	311	3931	529828

Rates per 100,000 women per year.

Cervical cancer accounts for 15% of all cancers in developing countries compared to 3.6% in developed countries. Overall, cervical cancer is relatively uncommon in the developed countries of the world, where intensive screening programs are in place. Since the advent and widespread use of screening Papanicolaou (Pap) smears, the incidence of cervical cancer has dramatically decreased. However, in many parts of the developing world, cervical cancer continues to cause significant morbidity and mortality .70% - 80% of human papilloma viral infections are cleared by the immune system within 6 to8 months and are not associated with any tissue abnormalities [15,16] .

Most HPV infections occur in young adults with a peak at 35-45 years of age. Currently, HPV infections have reached epidemic proportions in young, sexually active population. The mean age for women who develop cervical dysplasia is 25 years old, while carcinoma in situ and invasive cervical cancer has an older mean age of 30 and 50 years respectively [17,18].

1.3. Cervical cancer risk factors

The epidemiologic features associated with cancer of the cervix in women are low socioeconomic status, early age at the first intercourse, and multiple sexual partners. Besides, other risk factors, include age, ethnicity, multiparty, cigarette smoking and extend use of oral contraceptives, point to a venereal pattern of etiology [(19)]. Several risk factors increase women chance of developing cervical cancer. Women without any of these risk factors rarely develop cervical cancer[(20)].

Although these risk factors increase the odds of developing cervical cancer, many women with these risks do not develop this disease. When a woman develops cervical cancer or pre-cancerous changes, it may not be possible to confirm that a particular risk factor was the cause[(21)].

1.3.1.HPV-related risk factors

Human papillomavirus (HPV) is an important etiologic factor in the development of cervical intraepithelial neoplasia and the progression to cervical cancer [(22)]. There are important factors for the development of cervical carcinoma by HPV infection:

1.3.1.1. HPV Variant: HPV is a group of more than 200 related viruses . 30 types infect genital tract (types 16/18 most common),the types of HPVs are classified as high risk types (oncogenic or cancer-associated:16,18,31,33,35,39,45,51,52,56,58,59,68,82) and low risk types (non-oncogenic:6,11,40,42,43,44,54,61,72,73,81) [(23)]. Each HPV type acts as independent infection, HPV type16 and 18 cause 70% of cervical cancers and 50% of high grade cervical intraepithelial neoplasia. Different types of HPVs cause warts on different parts of the body. They may cause warts on or around the female and male genital organs and in the anal area. These warts may barely be visible or they may be several inches across. HPV type 6 and 11 cause most cases of genital warts. These are known as condyloma acuminatum [(24)] .

1.3.1.2. HPV Integration: The HPV DNA is usually extra chromosomal or episomal in benign cervical precursor lesions. However, cervical cancer tissues may contain both episomal and integrated HPV DNA at the same time. Although HPV integration appears to be rare in HPV- infected cells, its occurrence may also be an irreversible event that initiate a chain of events including the impairment of tumor suppressor genes (e.g., p53 and Rb), subsequent genomic instability and cell immortalization [(25,26)].

1.3.1.3. Viral Load: There has been recent interest in the association between HPV load and cervical disease. Although it is predictive, utility for identifying progression to cancer from HPV infection has yet to be determined [(27)].

1.3.1.4.Behavioural and Lifestyle Characteristic

Early onset of sexual intercourse especially if right after the first menses, multiple sexual partners and the sexual behaviour of the woman's male partner are risk factors for HPV infection [28,29].

1.3.2.HPV-cofactors and HPV- unrelated risk factors

1.3.2.1.Smoking

Cigarette smoking was associated with a two-fold statistically significant increase risk of cervical cancer with a significant dose response, and is an independent risk factor for cervical cancer [30].

Several mechanisms have been proposed for the association between smoking and cervical cancer. Smoking may affect cervical mucosal immune response and thereby increase risk of neoplasia [31].

Tobacco by-products have been found in the cervical mucus of women who smoke. These substances damage the DNA of cervix cells and may contribute to the development of cervical cancer [32].

1.3.2.2.Immunosuppression

HPV- associated cancers occur more frequently among patients with depressed cellular immunity, e.g. among immunosuppressed renal transplant recipients, among patients on immunosuppresive therapy and among HIV infected patients [33 – 35].

HIV infection impairs cell-mediated immunity and thus increase the risk of HPV-associated diseases, such as genital warts and malignancies. Latent HPV infection and squamous intraepithelial lesion (SIL) are much more common among HIV-infected women than HIV-negative women from the same populations [(36)].

HPV and HIV infections seem to interact synergistically to increase the risk of CIN, with some further mediation by the degree of immunosuppression [(37)].

1.3.2.3.Chlamydia infection

Chlamydia is a relatively common kind of bacteria that can infect the reproductive system. It spreads by sexual contact. Some studies have seen a higher risk of cervical cancer in women whose blood test results show evidence of past or current Chlamydia infection (compared with women who have normal test results) [(38)].

1.3.2.4.Diets

Women with diets low in fruits and vegetables may be at increased risk for cervical cancer. Also, overweight women are more likely to develop adenocarcinoma of the cervix[(39)].

1.3.2.5.Oral contraceptives (birth control pills)

There is evidence that taking oral contraceptives (OCs) for a long time increases the risk of cancer of the cervix. In a recent study, the risk of cervical cancer was doubled in women who took birth control pills longer than 5 years, but the risk returned to normal 10 years after they were stopped[40,41].

1.3.2.6.Multiple full-term pregnancies

Women who have had 3 or more full-term pregnancies have an increased risk of developing cervical cancer. No one really knows why this is true. One theory states that these women had to have had unprotected intercourse to get pregnant. So they may have had more exposure to HPV[42]. Also, studies have pointed to hormonal changes during pregnancy as possibly making women more susceptible to HPV infection or cancer growth. Another thought is that the immune system of pregnant women might be weaker, and thus allowing for HPV infection and cancer growth[43].

1.3.2.7.Young age at the first full-term pregnancy

Women who were younger than 17 years when they had their first full-term pregnancy are almost two times more likely to get cervical cancer later in life than women who waited to get pregnant until they were 25 years or older [44,45].

1.3.2.8.Poverty

Poverty is also a risk factor for cervical cancer. Many women with low incomes do not have ready access to adequate health care services, including Pap tests. This means that they may not get screened or treated for cervical pre-cancers[(46)].

1.3.2.9.Diethylstilbestrol (DES)

DES is a hormonal drug that was given to some women to prevent miscarriage between 1940 and 1971. Women whose mothers took DES (when pregnant with them) develop clear-cell adenocarcinoma of the vagina or cervix more often than would normally be expected. This type of cancer is extremely rare in non-DES exposed women . Still, there is no age cut-off when these women are safe from DES-related cancer. DES exposed women daughters may also be at increased risk of developing squamous cell cancers and pre-cancers of the cervix linked to HPV[(47,48)].

1.3.2.10.Family history of cervical cancer

Cervical cancer may run in some families. If the mother or the sister had cervical cancer, the chances of developing the disease are 2 to 3 times higher than the family without history [(49)].

1.4. Morphology of Cervical Cancer

There are three systems used to classify these cervical abnormalities:

- The World Health Organization classifies cervical dysplasia as mild, moderate, or severe as well as a separate category for carcinoma in situ (CIS) [(50)].
- The term cervical intraepithelial neoplasia (CIN) was introduced by Richart: CIN1 represents mild dysplasia; CIN2 is an intermediate grade; and CIN3, severe dysplasia or CIS[(50)].
- In the Bethesda system, a low grade squamous intraepithelial lesion (LSIL) corresponds to CIN1 and a high grade SIL (HSIL) encompasses both CIN2 and CIN3[(50)].

Mild dysplasia is called **CIN I** or flat condyloma. This lesion is characterized by koilocytotic changes mostly in the superficial layers of the epithelium- nuclear hyperchromasia and angulation with perinuclear vacuolization produced by cytopathic effect of HPV.

In **CIN II** , the dysplasia is more severe with the maturation of keratinocytes delayed into the middle third of the epithelium. It is associated with some variation in cell and nuclear size, heterogeneity of nuclear chromatin and mitoses above the basal layer, extending in to the middle third of the epithelium.

CIN III is marked by even greater variation in cell and nuclear size, chromatin heterogeneity, disorderly orientation of the cells, and normal or abnormal

mitoses. These changes affect virtually all layers of the epithelium and are characterized by loss of maturation[51.52].

1.5. Pathomorphology of HPV Infection

Four distinct but related forms of "pure" HPV infection have been identified[53,54]:

1. The papillary or exophytic form is rare on the cervix, but may be found on the vulva and the penis. There are marked papillomatosis, acanthosis and often keratinization of the superficial layers. The intermediate layers may contain koilocytes.
2. The flat condyloma (condyloma planum) which was described in 1977 displays koilocytes in the middle and upper layers of the epithelium. Often, there are blood vessels surrounded by scanty stroma reaching upwards towards the surface. There may be dyskeratosis, keratinization, or even epidermization.
3. The "spiked" condyloma is a variant of the flat lesion, characterized by small projections on the surface of the epithelium. These "spikes" contain a blood vessel surrounded by scarce stroma.
4. The "inverted" condyloma is rare. It may have a flat, spiked or papillary surface and grows inwards, replacing the columnar epithelium of the endocervical crypts. By far, the flat and "spiked" lesions are the more frequently seen. Mitotic figures vary in frequency. Even in "pure" HPV

infection, some mitoses may be atypical. The lesions can be polyploid or aneuploid.

1.6. HPV and intraepithelial Lesions

Morphological signs of HPV infection are frequently associated with the more advanced intraepithelial lesions. There are three distinct types of association[54,55]:

1. The horizontal association: Areas of intraepithelial lesions alternate with areas of HPV infection, either adjacent to each other or at a distance. Smears will contain both cells typical for HPV infection, and cells with more advanced changes correspond to the intraepithelial lesion. This is a common finding particularly well demonstrated on cone biopsies.

2. The vertical association: There is an intraepithelial lesion in which the upper layers display changes typical of HPV infection. The smear in such a case may only display the cells characteristic of HPV infection, scraped from the surface, while the more advanced lesion escapes detection. It is important to realize that this particular type of association may cause "false negative" reports.

3. The mixed association: In this lesion, changes characteristic of both HPV infection and of a more advanced lesion coexist within the same area and intimately intermixed. The smear will contain mostly dyskeratotic cells with

enlarged, darkly stained nuclei. The cellular pattern may be confused with that of an invasive keratinizing squamous carcinoma, and thus may cause "false positive" reports. This association had called the "atypical condyloma" previously but the term is best dropped.

1.7. Pathogenesis

1.7.1.The natural history of cervical cancer

The pathogenesis of cervical cancer is initiated by HPV infection of the cervical epithelium during sexual intercourse. Even though a high percentage of sexually active young women are exposed to HPV infections, only a very small percentage go on to develop cervical cancer [(56)].

Most women successfully clear the HPV infection through the action of a competent immune system. Approximately, 90% of lesions regress spontaneously within 12 to 36 months [(57)].

Other factors, such as genetic predisposition, frequency of reinfection, intratypic genetic variation within HPV type, coinfection with more than one HPV type and hormone levels may also influence the ability to clear an HPV infection[(58)].

The importance of the host immune system in preventing the development of cervical carcinoma comes from the analysis of HPV infections in human immunodeficiency virus (HIV)-positive women. HPV infections with high-risk viral types, persistence of HPV infection and the presence of squamous intraepithelial

lesions are more common within this immunocompromised group than in immunocompetent women [58].

The host cellular immune response is mediated by cytotoxic T cells and requires the interaction of viral epitopes with histocompatibility class I molecules. A humoral immune response also develops, but local levels of HPV-specific immunoglobulin G (IgG) and IgA in tissue do not correlate with clearance of virus [59].

However, systemic levels of HPV-specific IgA have been correlated with virus clearance. In contrast, systemic levels of HPV-specific IgG have been detected more frequently in patients with persistent HPV infection[59] .

The natural history of cervical cancer is a continuous disease process that progress gradually from mild cervical intraepithelial neoplasia (CIN) to more severe degrees of neoplasia (CIN 2 or CIN 3) and finally to invasive cancer [60].

Mild and moderate dysplasias are associated with continued viral replication and virus shedding, and most of these lesions spontaneously regress. Progression to high-grade lesions and ultimately invasive cancer is usually associated with conversion of the viral genome from an episomal form to an integrated form, along with inactivation or deletion of the E2 region and expression of the E6/E7 product genes (Figure 1.1) .

Progression to cancer generally takes place over a period of 10 to 20 years. Some lesions become cancerous more rapidly, sometimes within two years [60].

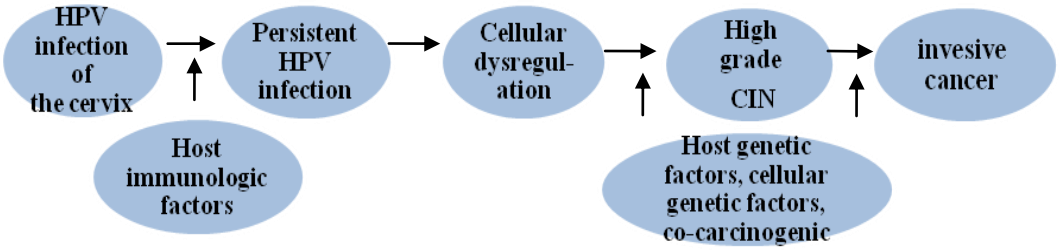

Figure 1.1: Model for the development of cervical carcinoma [58].

1.7.2. molecular basis

Basal cells of stratified squamous epithelium may be infected by HPV. Other cells types appear to be relatively resistant. The HPV replication cycle begins with entry of the virus into the cells of the basal layer of the epithelium through mild abrasion or micro-trauma of the surface epithelium. Once inside the host cell, HPV DNA replicates progress to the surface of the epithelium[61].

In the basal layer, viral replication is considered non-productive, and the virus establishes itself as a low-copy number episome by using the host DNA replication machinery to synthesize its DNA on average once per cell cycle[62].

In the differentiated keratinocytes of the suprabasal layer of the epithelium, the virus switches to a rolling-circle mode of DNA replication, amplifies its DNA to high copy number, synthesizes capsid proteins, and causes viral assembly [63].

High-risk HPV types can be distinguished from low-risk HPV types by the structure and function of the E6 and E7 products. In benign lesions caused by

HPV, viral DNA is located extrachromosomally in the nucleus. In high-grade intraepithelial neoplasias and invasive cancers, HPV-DNA integrated generally is into the host genome. The integration of HPV-DNA disrupts or deletes the E2 region, and therefore results in loss of its expression [(64)].

This interferes with the function of E2, which normally down-regulates the transcription of the E6 and E7 genes, and leads to an increased expression of E6 and E7 genes. The function of the E6 and E7 products during a productive HPV infection is to subvert the cell growth-regulatory pathways and modify the cellular environment cycle by binding and inactivating two tumor suppressor proteins: the tumor suppressor protein (p53) and the retinoblastoma gene product (pRb) in order to facilitate viral replication [(65,66)].

The HPV E6 gene product binds to p53.The normal activities of p53 which govern G1 arrest, apoptosis, and DNA repair are abrogated. The HPV E7 gene product binds to pRb and this binding disrupts the complex between pRb and the cellular transcription factor E2F-1,resulting in the liberation of E2F-1. This allows the transcription of genes whose products are required for the cell to enter the S phase of the cell cycle [(63)]. The E7 gene product can also associate with other mitotic ally interactive cellular proteins, such as cyclin E. The outcome is stimulation of cellular DNA synthesis and cell proliferation [(66)] (Figure 1.2) .

Next, the E5 gene product induces an increase in mitogen-activated protein kinase activity, thereby enhancing cellular responses to growth and differentiation factors. This results in continuous proliferation and delayed differentiation of the host cell [(66)].

The inactivation of p53 and pRb proteins can give rise to an increased proliferation rate and genomic instability. As a consequence, the host cell accumulates more and more damaged DNA that cannot be repaired, leading to transformed cancerous cells [(67)].

In addition to the effects of activated oncogenes and chromosome instability, potential mechanisms contributing to transformation include methylation of viral and cellular DNA, telomerase activation, and hormonal and immunogenetic factors [(67)].

Low-risk HPV E6 proteins do not bind p53 at detectable levels and have no effect on p53 stability in vitro. The E7 protein from low-risk HPV types binds pRb with decreased affinity[(67,68)].

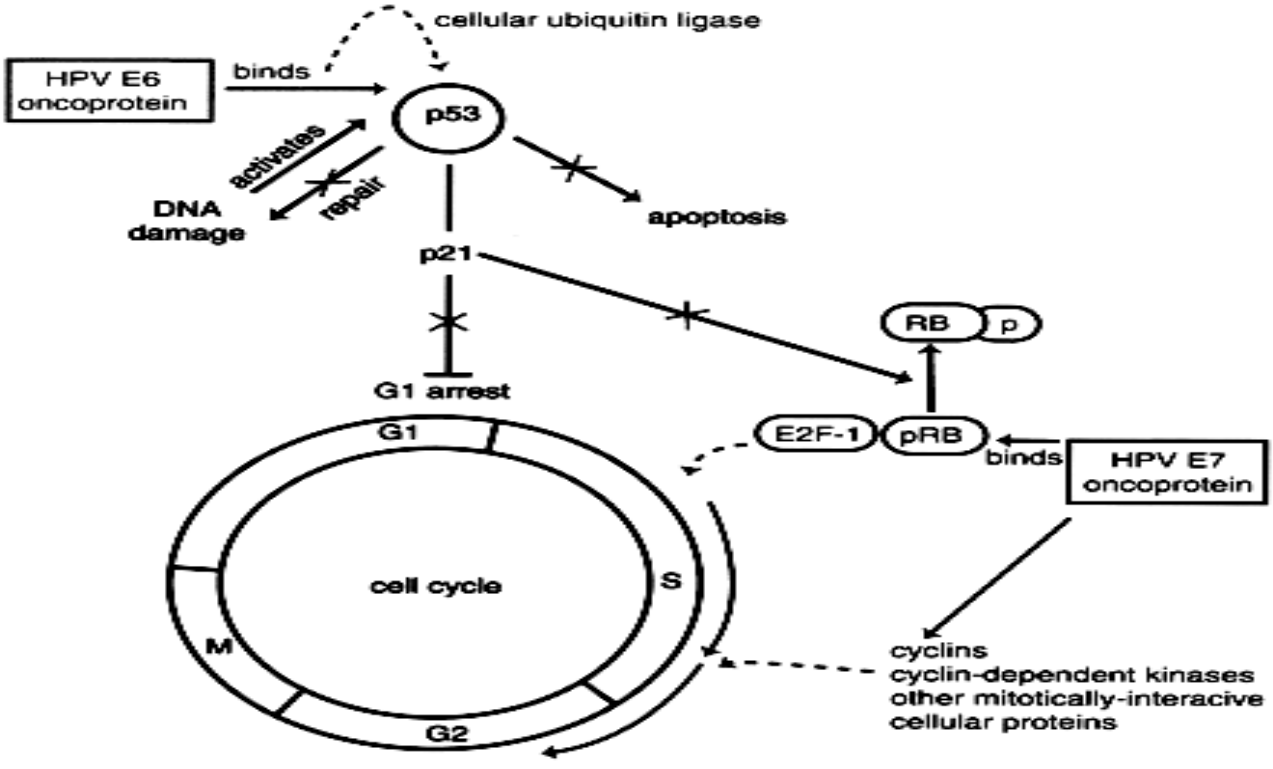

Figure 1.2: Molecular mechanisms of oncogenic HPV infection [(61)].

1.8. Signs and symptoms of cervical cancer

Women with early cervical cancers and pre-cancers usually have no symptoms. Symptoms often do not begin until the cancer becomes invasive and grows into nearby tissue[(69)]. When this happens, the most common symptoms are:

- Abnormal vaginal bleeding, such as bleeding after sex (vaginal intercourse), bleeding after menopause, bleeding and spotting between periods, and having (menstrual) periods that are longer or heavier than usual. Bleeding after douching, or after a pelvic exam is a common symptom of cervical cancer but not pre-cancer[(70)].
- An unusual discharge from the vagina ,the discharge may contain some blood and may occur between a menstrual periods or after menopause.
- Pain during intercourse.

These signs and symptoms can also be caused by conditions other than cervical cancer. For example, an infection can cause pain or bleeding. Still, if you have any of these signs or other suspicious symptoms were present health care professional should be seen right away. Ignoring symptoms may allow the cancer to progress to a more advanced stage and lower the chance for effective treatment. Even better, women must not wait for symptoms to appear. They must have regular Pap tests and pelvic exams[(70,71)].

1.9.Types of cervical carcinoma

The World Health Organization (WHO) recognizes two main histological types of invasive cervical carcinoma [(72,73)]:

- Squamous cell carcinoma (which constitute about 85% of all cases)

- Keratinizing (well differentiated and moderately differentiated)
- non keratinizing (large and small cell types)
- Spindle cell carcinoma

- Adenocarcinoma (which constitute about 10-12% of all cases)
  - Variant : adenoma malignum (minimal deviation carcinoma)
  - Variant :villoglandular papillary adenocarcinoma

other types of cervical carcinoma make up the remaining 3-5%of all cases: Endometrioid adenocarcinoma , Clear cell adenocarcinoma, Serous adenocarcinoma , Mesonephric adenocarcinoma , Intestinal type (signet ring) adenocarcinoma, adenosquamous carcinoma, adenoid cystic carcinoma, Small cell carcinoma , Undifferentiated carcinoma and Metastatic tumors (breast, ovary, colon, and direct spread of endometrial carcinoma) . Squamous and adenocarcinoma may actually have distinct causes despite being so close together anatomically. Most risk factors are indeed common to both types.

Four main features differentiate their epidemiologic and prevention characteristics:

1. Unlike squamous carcinomas, the incidence of adenocarcinoma has been increasing in recent years particularly among younger women and other developed nations[(74)].

2. HPV 16 is the HPV type most frequently found in squamous carcinomas, whereas HPV 18 is found in more than half of adenocarcinomas[(75)] **.**

3. Increased parity is associated with an increased risk of squamous but not adenocarcinomas[(76)]

4. Pap cytology is not as efficacious in detecting adenocarcinomas. New screening technologies need to take into account the need to incorporate more sensitive methods for detecting adenocarcinoma[(76)]

Very rarely, other types of cancer can occur in the cervix. For example, lymphoma is a cancer of the lymphatic system[(77)].

1.10.Stages of cervical cancer

Most of staging systems depend on the size of tumor, number of lymph nodes involved by the tumor , and tumor metastasis. Recently, the TNM staging system has become the most commonly used [(78)]. This staging system contains several substages, but basically categorizes tumors using the following scale (Table 1.3):

Table 1.3 :TNM staging system of cervical carcinoma[(78)]

STAGE	TNM	DESCRIPTION
0	Tis N0 M0	In situ
I	T1 N0 MO	Confined to cervix
IA	T1a N0 M0	Diagnosed only by microscopy
IA1	T1a1 N0 M0	Depth ≤3 mm, horizontal spread ≤7 mm
IA2	T1a2 N0 M0	Depth >3-5 mm, horizontal spread ≤7 mm
IB	T1b N0M0	Clinically visible or microscopic lesion, greater than T1a2
IB1	T1b1 N0M0	≤4 cm
IB2	T1b2 N0M0	>4 cm
II	T2 N0M0	Beyond cervix but not pelvic wall or lower third vagina

IIA	**T2A N0M0**	**No parametrium involvement**
IIB	**T2b N0M0**	**Parametrium involvement**
III	**T3 N0M0**	**Lower third vagina/pelvic wall/hydronephrosis**
IIIA	**T3a N0M0**	**Lower third vagina**
IIIB	**T3b N0 M0 or N1M0**	**Pelvic wall/hydronephrosis**
IV	**T4**	**Mucosa of bladder/rectum; beyond true pelvis**
IVA	**T4a any N M0**	**organs close to the cervix, spread to the bladder or rectum.**
IVB	**T4b any N M1**	**spread to distant organs beyond the pelvic area, such as the lungs or liver.**

T, tumor size. N:Regional Lymph Nodes, N0: No regional lymph node metastasis, N1: Regional lymph node metastasis. M:Distant Metastasis, M0:No distant metastasis, M1: Distant metastasis.

1.11.Grades of cervical cancer

To find out the grade of a tumour, the biopsy sample is examined under a microscope.A grade is given based on how the cancer cells look and behave compared with normal cells. This can give the healthcare team an idea of how quickly the cancer may be growing[(79)]. There are three grades (Table 1.4):

Table 1.4: Histological Grading of cervical carcinoma[(79)].

GRADE	DESCRIPTION
I	Low grade – slow growing, less likely to spread
II	Moderate grade
III	High grade – tend to grow quickly, more likely to spread

1.12. Prognosis

Prognosis depends on the stage of the cancer, a person's general health, how well the cancer responds to treatment, previous outcomes of large numbers of people who had the disease.

The 5-year survival rate refers to the percentage of patients who live at least 5 years after their cancer is diagnosed. Many people live much longer than 5 years and many are cured.

With treatment, the 5-year relative survival rate for the earliest stage of invasive cervical cancer is 92%, and all stages combined is about 72%. These statistics may be improved when applied to women newly diagnosed[(80)].

With treatment, 80 to 90% of women with stage I cancer and 50 to 65% of those with stage II cancer are alive 5 years after diagnosis. Only 25 to 35% of women with stage III cancer and 15% or fewer of those with stage IV cancer are alive after 5 years. Survival improves when radiotherapy is combined with chemotherapy[(81)] (Table 1.5).

The numbers below come from the National Cancer Data Base, and are based on people diagnosed between 2000 and 2002.

Table 1.5: 5-Year Survival Rate of cervical carcinoma[80,81].

STAGES	5-YEAR SURVIVAL RATE
0	93%
1	80%-93%
2	58%-63%
3	32%-35%
4	15%-16%

2.1. Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing proteins in cells of a tissue section utilizing the principle of antibodies binding specifically to antigens in biological tissues [82]. IHC is widely used in basic research to understand the distribution and localization of biomarkers in the tissues. However, it is also widely used in the diagnosis and treatment of cancer because specific molecular tumor markers are characteristic of particular cancer types[83].

2.1.1.Tumor markers

Tumor markers are substances that can be detected when their levels increased higher than normal in blood, urine, or tissues of some patients with certain types of cancer. They include cell surface antigen, cytoplasm protein, enzymes, and hormones, and can be divided into: tumor-derived markers which are produced by the neoplastic cells and tumor-associated or host response markers which include metabolic and immunological products of normal tissue in response to the presence of neoplastic cells[(84)]. These markers are used as antigens that can be identified using antibodies to locate them in tissues. These antigens which are localized in the cytoplasm, nuclei, or to the cell surface can be visualized by the light microscopy[(85)].

Immunohistochemical techniques can detect tumor markers in fresh, frozen, and paraffin embedded tissue biopsies. Most of the specimens stained in a routine immunohistochemistry laboratories of surgical pathology are formalin-fixed, paraffin embedded tissues. Fixation and embedding cause antigen masking, but also better retention of labile proteins, nucleic acids and small peptides. In order to overcome the drawback of antigen loss, enzymatic- or heat- mediated antigen retrieval is used [(86)].

2.1.2.The antibodies

The antibodies can be polyclonal or monoclonal. Monoclonal antibodies are generally considered to exhibit greater specificity, but with low or moderate sensitivity and more cost than polyclonal antibodies.

However, polyclonal antibodies have higher affinity and wide reactivity but lower specificity when compared with monoclonal antibodies. The most common antibody used in immunohistochemistry is Immunoglobulin G while Immunoglobulin M is much less commonly used [(86)].

2.1.3.The principles

The immunodetection of antigens is a two-step process: the binding of an antibody to specific requested antigen, and the detection and visualization of a bound antibody by one of a variety of enzyme chromogenic systems. The choice of detection system will dramatically impact on sensitivity, utility, and ease of use of the method[(87)], so visualization methods are divided into[(88-90)] :

1. Direct method is primarily used for immunoglobulin deposits. It is easy and fast but insensitive and only few available antibodies are used. The primary antibody is often labelled with a flourochrome. An example of this type is EPOS (Enhanced Polymer One Step).

2. Indirect method involves application of secondary antibody. There are several techniques:

APAAP (Alkaline Phosphatase Anti-Alkaline Phosphatase) is performed as three steps by incubation with primary antibody, application of secondary antibody and then by the application of APAAP complex.

PAP(Peroxidase Anti-Peroxidase) consists of soluble immumocomplexes of peroxidase and high-avidity antibody to peroxidase. It also consists of 3 steps.

ABC (Avidin-biotin complex) which involves the application of biotin-labeled secondary antibody is followed by the addition of avidin-biotin-peroxidase complex and gives a superior result when compared to the unlabeled antibody method.

EnVision+ systems (2-step polymer based method). They are easy, fast, sensitive and can stick to the tissue, and based on unique enzyme-conjugated polymer backbone, which also carries secondary antibody molecules. Thirty minutes incubation time with primary antibody and EnVision reagent respectively is recommended.

LSAB^{+} method (Labeled Streptavidin Biotin Peroxidase Complex technique) Streptavidin is derived from streptococcus avidini, which is a recent innovation for substitution of avidin.

Many reports suggested that LSAB method is about 5 to 10 times more sensitive than ABC method, the principle of which is described in the following steps[(91)]: (Figure 1.3)

Step1: The application of primary antibody, which is a monoclonal antibody binds with specific tissue antigen.

Step2: The application of secondary (link) antibody.This antibody binds to the primary one, the secondary antibody is produced in another species (e.g.mouse) that recognizes the primary antibody as antigen. Additionally, this antibody has biotin molecule attached to it.

Step3: The application of label. It consists of Streptavidin in stead of avidin attached to the enzyme Peroxidase, which has an affinity to Biotin. This will provide an enzyme (Peroxidase) at the site of antibody- antigen reaction.

Step4: The application of chromogen-substrate.The enzyme peroxidase acts on its substrate hydrogen peroxide (H_2O_2) producing oxygen radicals, which oxidizes the chromogen DAB (diaminobenzedin) producing brown color.

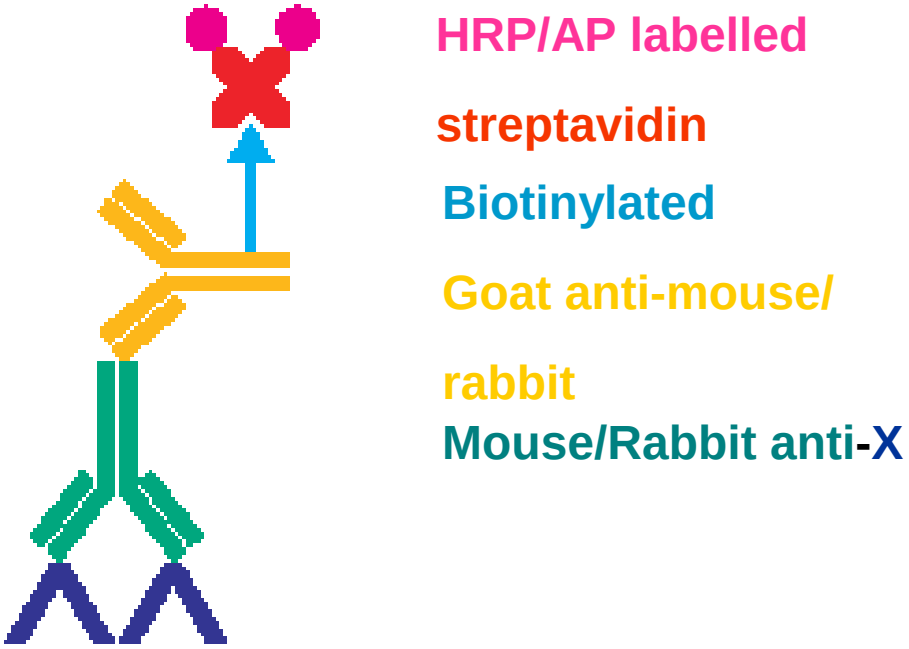

Figure 1.3: Diagram showing LSAB$^+$ method.

2.2. Chromogenic In Situ Hybridization

A new ISH technique is based on chromogenic signals instead of fluorescent signals. The technique, chromogenic in situ hybridization (CISH), has proven qualities with respect to ease of use that might explain the recent growth in popularity[92-94]:

1. CISH results are easily interpreted by the use of a bright-field microscope which is generally used in diagnostic laboratories.

2. CISH enables visualization of the nucleus and is also able to distinguish invasive from in situ carcinomas.

3. CISH signals do not generally fade over time allowing the tissue samples to be archived and reviewed later.

4. CISH resembles IHC to a large extent (as opposed to FISH) due to the use of conventional counter stains, e.g. hematoxylin, for visualization of tissue morphology.

Because CISH combines the genetic information from FISH with the visualization and interpretation resembling IHC, the CISH technique is a practical and used friendly alternative to FISH.

2.2.1.Antibodies

In situ hybridization method use label probe (biotinylated DNA probe) to localize specific nucleic acid sequences at sub-cellular levels.It enables visualization of the nucleus and is also able to distinguish invasive from in situ carcinomas[95].

2.2.2. The Principles

Chromogenic visualization (colorimetric method) is based on enzyme-conjugated antibodies that recognize the target of interest (specific nucleic acid at sub-cellular level. Reaction of substrates with enzymes such as horseradish peroxidase (HRP) and/or alkaline phosphatase (AP) leads to chromogen precipitates, which then can be detected with a bright field microscope[(96)].

A biotinylated probe hybridizes to the target sequence (HPV DNA), and then an anti-biotin antibody binds to the biotin on the hybridized probe. In order to re – introduce biotin into the system, an immunoglobulin that is coupled with substantially higher numbers of biotin moieties than the probe binds to the antibody. These additional layers initiate amplification of the signal. To visualize the antibody/probe complex, streptavidin – alkaline phosphatase conjugate is added. Each molecule of conjugate contains up to alkaline phosphatase molecules, providing further amplification of the reaction[(97)].

2.2.3.GenPoint Tyramide Signal Amplification System for Biotinylated Probes

Tyramide Signal Amplification (TSA) is a peroxidase-based signal amplification system which is compatible with all in situ hybridization as well as immunocytochemical detection systems. TSA is an extremely powerful signal amplification system that boosts hybridization signals up to 1000 fold. After amplification, additional steps are needed to give a detectable signal.

Biotin is commonly used to label probes for non-radioactive detection[(98-102)]**.** Standard biotin detection schemes utilize enzyme conjugates of streptavidin to produce precipitating dye signals from chromogenic enzyme substrates. The

GenPoint system creates an additional level of amplification for biotin detection. After an initial binding of streptavidin-peroxidase to the biotinylated probe, the peroxidase catalyzes the oxidation of biotinyl tyramide[(103)], which immediately forms covalent bonds with aromatic groups in the specimen. This reaction deposits large amounts of biotin at the site of hybridization. The additional biotin is then used to capture more streptavidin-peroxidase. This amplification cycle of streptavidin-peroxidase > biotinyl tyramide > streptavidin-peroxidase may be repeated to further increase the accretion of biotin. The signal is finally developed by adding the chromogenic indicator dye diaminobenzidine (DAB), which is oxidized by the peroxidase enzymes to produce a dark brown precipitate at the site of hybridization[(103)].

2.3.HPV Structure

Papillomavirus particles, regardless from where they are isolated, are very similar in appearance .

A. Papillomaviral Particles

HPV virions are small, non – enveloped, approximately 45-55 nm in diameter with an icoshedral capsid composed of 72 capsomers [(104,105)]. Virions consist of a central core of DNA and enclosed within an outer capsid of viral protein that is arranged in a symmetrical 20-sided pattern that seems almost spherical on electron

microscopy. The virion does not have an outer membrane which may, in part, account for the low antigenicity of papillomavirus infection[106]. The capsid consists of two structural proteins: L1, which is the group antigen; and L2, which is thought to be type – specific [107].

B. Papilloma viral Genome

The HPV genome consists of single molecule of double – stranded circular DNA approximately 8000 bp in size. The intact DNA is supercoiled and thus resembles an endless loop of twisted telephone handset cord. The viral DNA is packaged in and around protein from the cell nucleus, (i.e. histones and associated peptides), into a structure that resembles cellular chromatin. HPV genome has molecular weight of about 5.2 x 106 Daltons [108-110].

All open reading frames (ORF) are located on the same DNA strand, although some involve overlapping sequences in different reading frames. While the other strand is assumed to be non–coding, the demonstration of anti–sense transcripts in cervical cancer tissue suggests that it may have regulatory role .The genetic information is divided to three major regions: two protein–encoding regions, early (E) and late (L) region and a non-coding upstream regulatory region (URR) or long control region (LCR) that contains the origin of replication and many of the control elements for transcription and replication. Within the early and late regions, it is possible to distinguish different genes with specific functions[111-112] (Table 1.6) .

Table 1.6: Functions assigned to the papillomavirus .

Gene Category	Genes	Major Function
Early genes	E1	Viral replication
	E2	Modulation of transcription and replication
	E3	Unknown
	E4	Productive viral infections
	E5	Transforming properties
	E6	Oncoprotein; interaction with p53 protein
	E7	Oncoprotein; interaction with pRb protein
	E8	Unknown
Late genes	L1	Major capsid protein
	L2	Minor capsid protein

2.4.Immune Response to HPV Infection

Infection by HPV causes dysplastic lesions in the cervical epithelium. In the great majority of cases, these lesions are self limiting within 6 to 8 months, demonstrating effective host immune response to the virally infected cell. However, the virus is not cleared in a small group of women and the infection becomes persistent. Deficient intracellular surveillance of persisting

papillomavirus infection was proposed as a possible mechanism of the development of human genital cancer .

The first line of defense at the mucosal surface against infection is the innate immune response. Natural killer (NK) cells induce apoptosis in virally infected cells and in tumor cells . Down regulation of HLA class I molecules is frequently seen in cervical cancer leading to the activation of NK cell and cytolysis [113-115].

The antibody response to HPV does not seem to affect regression or persistence of cervical lesions. These antibodies, particularly those that target the proteins comprising the virion capsid (L1 and L2 proteins), might be effective at preventing infection. Antibodies to both HPV capsids and several other HPV antigens are seen simultaneously within a few months following acquisition of HPV infection [116,117].

The role of the immune system in the viral clearnce is not clear, but there is circumstantial evidence that Cell Mediated Immunity (CMI) is important in the control of persistent HPV infection [118]:

1- HPV associated cervical lesions are more prevalent in immunosuppressed individuals.
2- Spontaneous regression of HPV–induced genital warts is associated with an infiltration of lymphocytes into the lesion.
3- The presence of lymphocytes in the lesion correlates with improved prognosis in squamous cervical cancer (SCCs) .

2.5. Mechanistic Role for HPVs in Cervical Neoplasia

A. DNA Integration

In premalignant cervical lesions, the HPV genome is typically maintained in its episomal form. However, the majority of invasive cervical carcinomas contain HPV DNA that has integrated into the host cell genome. Thus, the integration event is temporally associated with the acquisition of the malignant phenotype [(119,120)]. The oncogenic HPV DNA preferentially integrate into common chromosomal fragile regions [(121)]. In invasive genital cancers, the E6-16 and E6-18 genes have been found to be integrated in chromosome near the c-*myc* proto-oncogene.

The E2 function is lost during carcinogenic progression as a result of the viral DNA integration into the cellular genome and the concomitant disruption of the E2 open read frame (ORF) and increase the level of E6 and E7 [(122-124)]. However, the overexpression of E6 and E7 is only sufficient to immortalize primary epithelial cells. Thus, additional human mutation need to take place in order to transform the cells [(125)].

The causal relationship between HPV infection and cervical cancer is primary due to the ability of the vial oncoproteins, E6 and E7, to abrogate the cell cycle [(126,127)].

B. E6-p53 Model

p53 is 53-KD a nuclear phosphoprotein which is encoded by a tumor-suppressor gene and located on the short arm of chromosome 17. Wild type p53 protein has been shown to have a central role in regulation of the cell cycle and apoptosis [(128,129)]. Also, p53 is a transcription factor. It stimulates the expression of genes

involved in cell cycle arrest and apoptosis, such as the cyclin-dependent kinase inhibitor p21CIP-1 [130].

In cervical carcinoma, there are two type of alteration of p53 expression; inactivation of wild type p53 by binding with the protein product of E6 gene of high-risk HPV, and less commonly spontaneous mutation of p53 in HPV negative tumours [131-133].

The E6 oncoprotein is able to form complexes with the wild-type p53 protein, leading to functional inactivation and rapid degradation[134].

E6 binding to p53 can also lead to retention of p53 in the cytoplasm and block its translocation to the nucleus and hence abrogating its function independently of degradation . The cells in which the p53 is inactivated lose the capacity to arrest the cell cycle in G1 phase or the mechanism of apoptosis, resulting in accumulation of genetic mutation which could eventually lead to malignancy [135].

The tumorigenic properties of E6 and E7 proteins may not necessarily be limited only to the Rb- and p53-related pathways[136]. E6 oncogene of malignancy-associated HPVs increases cellular telomerase activity, predominantly via transcriptional activation of the catalytic subunit of telomerase. Telomerase is an enzyme that restores telomeric DNA sequences, and the expression of its activity is thought to be involved in immortalization of human cells in-vitro and eventually tumor progression in-vivo [137-139].

C. E7-pRb-p16INK4a Model

pRb is a nuclear protein of 105 to 110 KDa and its locus is located on chromosome 13q 14 . Rb protein family members play a pivotal role in eukaryotic cell cycle regulation. Hypophoshorylated Rb binds to transcription factors of the E2F family and represses the transcription of particular cell cycle genes. When cell progresses in mitosis from G0 through G1 to S-phase, Rb gets hyperphosphorylated by G1 cyclin-cyclin dependent kinases, releasing the transcription factor E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression. Rb remains phosphorylated during S, G2, and M, until late M, when it is dephosphorylated by a specific phosphatase[(140)].

The HPV E7 gene product binds to the hypophosphorylated form of the Rb family of proteins. This binding results in phosphorylation of these proteins, in their enhanced degradation, and in the release of transcription factors of the E2F family, activating transcription of gene regulating cell proliferation [(141)]. E7 proteins of high-risk HPVs are found in cyclin E and in cyclin A complexes. These complexes exhibit kinase activity. The activation of cyclin E, followed by the activation of cyclin A, is mediated by E7 sequences required for transformation. Also, an important function of E7 proteins is the inactivation of the cyclin - dependent kinase inhibitors p21CIP-1 and p27KIP-1. This interaction uncouples cdk activity from cdk inhibitors and should be a major factor in growth stimulation of HPV-infected cells [(142)].

Another tumor suppressor protein involved during G1/S phase is the p16 INK4a. This protein, encoded by the CDKN2A (INK4A) tumor suppressor gene on chromosome 9 p 21, inactivates the function of cdk4- and cdk6-cyclin D complexes [(143)]. Functional loss of p16 has been reported for many human cancers, whereas in HPV-associated cervical carcinomas p16 overexpression has

been observed. As the HPV E7 oncogene product inhibits the activity of the pRb protein, p16 is up-regulates via the loss of the negative feed back control of pRb expression[144,145]. The p16 transcription may also be directly induced by the transcription factor E2F released from pRb after binding of the viral oncoprotein E7. It could be demonstrated that p16 is a specific biomarker that can routinely be used to identify dysplastic cervical epithelia. Furthermore, p16 immunostaining has been shown to identify lesions with relevant viral oncogen expression[145,146] .

The E7 protein binds to numerous other cellular proteins, but the physiological consequences of these interactions are largely unknown. Also recently, HPV-16 E7 has been shown to induce abnormal centrosome duplication resulting in multipolar, abnormal mitoses and aneuploidy. Although E6 can potentiate this effect, E7 plays major role in inducing these centrosome related mitotic disturbances, the mechanism by which E7 affects centrosome homeostasis is not yet known, Abnormal

centrosome duplication induced by HPV E7 rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell.

2.6. Diagnosis of HPV Infection and Cervical Neoplasia

2.6.1.Conventional Cytology

The Pap smear (i.e., sampling of exfoliated cells from the uterine cervix for microscopic examination to detect underlying cancer or precursors) was developed by George Papanicolaou in 1943 . Since its introduction and

widespread use, deaths from cervical cancer in the United States have been reduced by almost 75% [147].

The primary goal of cervical cytologic screening is to identify women in whom further evaluation with colposcopy is required to detect the presence of true cancer precursors [148].

The Pap smear has been a 20-30% false–negative rate for detecting cervical dysplasia [1148,149]. False – negative results arise for two primary reasons, sampling errors and laboratory erros in screening and interpretation [150-152].

2.6.2.New Directions in Cervical Cancer Screening

1.Computerized Screening

Because human fatigue and error may be major contributors to false-negative reading of Pap smear, computer-assisted image analysis and artificial intelligence have been introduced as a means of improving the sensitivity of Pap smear [153].

2-Liquid-Based Sampling Techniques

Recently, a new liquid-based approach to cervical cytology sampling was introduced. Cervical cells are rinsed directly into a viral containing a fixative and then filtered from blood, mucus and inflammatory cell. A monolayer of cells is then placed on the glass slide for staining and manual screening. This technology aims to decrease false-negative results by optimizing the collection and preparation of cervical cells [154].

2.6.3.Visual Inspection of the Uterine Cervix

Visual inspection of the cervix after application of 3-5% acetic acid (VIA) and visual inspection of the cervix after application of 2% Lugol's iodine(VILI) are simple, inexpensive, real-time availability of result but the major disadvantage of these tests is the low specificity[155].

2.6.4.Colposcopic Follow-up of Abnormal Pap Smear

HPV cytopathy or cervical dysplasia should be further evaluated by colposcopy, colposcopic directed biopsy and endocervical curettage ,the colposcopic magnifies the epithelium from 4 to 40 times, enabling visualization of epithelial and vascular changes typical of low-grade and high-grade dysplasia and cancer[156].

Colposcopy showed high degree of sensitivity and specificity (96% and 99.2%) in case of carcinoma of cervix, 82.8% sensitivity and 49.6% specificity in CIN, and 93.7% specificity and poor sensitivity (19.7%) in subclinical papillomavirus [157].

2.6.5.Electron Microscopy for Diagnosis of HPV Infection

Electron microscopy can be used on tissues obtained by colposcopically directed biopsies, or on cells reprocessed from Papanicolaou stained cell spreads. The virion is localized within the nuclei of koilocytes or dyskeratocytes. However, its use has been almost exclusively confined to the research laboratory and it currently has little role in routine diagnosis[158].

2.6.6.Immunology in Diagnosis of HPV Infection

1.Immunohistochemical Staining

Immunohistochemical techniques have been in use as adjunctive methods for the recognition of cells in tissue sections for at least 50 years. The first method reported the use of fluorescent labels in 1941 [(159)]. In 1974, reports of using enzymatic labels; such as horseradish peroxidase (HRP), in routine H & E paraffin sections appeared [(160)]. The methods have since then became the "standard of car" in surgical pathology when classic methods alone failed to yield a definitive diagnosis [(161)]. Early techniques were based on the peroxidase-anti-peroxidase (PAP) reaction, while improvement exploited the strong affinity of avidin for biotin. The anti-serum is applied at a suitable dilution to histological (Immohistochemistry) or cytological (Immunocytochemistry) preparations, and then the site of the bound antibody is demonstrated by one of a number of techniques resulting in the deposition of a coloured product which can be seen by routine microsocopy. Monoclonal antibodies have, however, been produced that could have diagnostic potential. These are mainly antibodies directed against genetically engineered L1 fusion proteins, although monoclonal and polyclonal antibodies directed against L2, E2, E4, E5, E6 and E7 have also been studied .The classic method has the advantage to demonstrate presence of viral particles (viral capsid antigen) in relation to the morphology of the tissue in histological section from paraffin embedded tissue specimens. Capsid proteins are not usually found in cervical cancer, since invasive cancers are not permissive for HPV replication. Therefore, immunohistochemistry is mostly useful for the study of HPV infectivity and spread[(162,163)] .

In vitro studies have shown that for the initation and maintenance of the malignant phenotype, the expression of the HPV- transforming protein E6 is required. Thus, HPV infection defined by the presence of HPV DNA may not be sufficient to evaluate tumor progression. E6 protein expression would, therefore, be a necessary requirement for HPV activity [(164)].

2.Serology

In epidemiological studies, HPV serology using virus capsids is a useful method to measure past and present infections, that is, the lifetime exposure to HPV. IgG marks lifetime cumulative HPV exposure, whereas IgA marks recent or ongoing infections. The major benefits of HPV capsid serology are high specificity, a simple and easily standardized assay and its potential to also measure past infections. The disadvantage is the moderate sensitivity . HPV is inherently only weakly antigenic and immune response to the virus is quite variable among individuals. In addition, viral subtyping is problematic with these techniques, since the capsid proteins of different HPV subtypes are antigenically quite similar [(165,166)].

Antibody levels against early proteins and peptides (E2, E4, E6, and E7) can be used to predict outcome in follow-up studies of CIN and cancer patients. However, these associations are not strong enough to be clinically useful . Although serological surveys of HPV infection have been published, the detection of antibody to HPV is not a useful diagnostic test, because of a significant false negative rate [(166,167)].

2.6.7.Molecular Diagnosis of HPV Infection

1. Polymerase Chain Reaction (PCR)

The most sensitive technique available is the polymerase chain reaction. It can detect one HPV genome in 105 cells. It has been applied to the detection of HPV in many different materials including fresh biopsy, cervical scrapes "cytobrushing" and paraffin embedded tissue [(168)]. PCR is used frequently as a diagnostic tool in epidemiologic investigation of HPV, but the associated costs and technology requirements are often inappropriate for large screening program [(169)].

2. In Situ Hybridization (ISH)

In situ hybridization, techniques have become important tools to detect nucleic acid taget sequences . This method has been widely used for the detection of HPV nucleic acid with either isotopic or non-isotopic probes. The possibility of using non-radioactive labels has made this technique more attractive for diagnostic laboratories because it avoids problems relative to the short life of radioactive compounds, their disposal, and personnel safety. The most commonly used non-isotopic labels for in situ hybridization are digoxigenin, biotin, and fluorescein, which can be visualized with final enzymatic reactions using colorimetric substrates. Nonisotopic techniques are fast and give a precise localization of the hybridization product, but the drawback is its low sensitivity. However, the sensitivity is dependent on the detection system used [(170,171)].

It is an easy to handle, reliable method for HPV detection and typing, working on both Pap-smear and paraffin-embedded sections. The main advantage of the

in situ format is the ability to correlate DNA probe results with cellular morphology . Its main problem so far now was its low sensitivity, with a detection limit of 10 to 50 viral copies per cell in formalin-fixed samples[172,173]. Therefore, several attempts have been undertaken to enhance the sensitivity of ISH. Signal-amplified in situ techniques have been developed to detect a small number of HPV nucleic acid sequences with high sensitivity by using a biotinylated probe for immunohistochemical detection of HPV in formalin-fixed, paraffin-embedded biopsy tissue sections. This assay is able to detect as few as 1 to 2 copies of target sequence per nucleus [174].

3. Southern Blot DNA – Hybridization

This is the "gold standard" method of detecting the HPV genome under ideal condition. It can detect one copy of the genome in 10-100 cells by depending on factors such as the amount of total cellular DNA and activity of the probe label. An additional feature of southern blotting is that it provides information about the physical state of viral genome: whether it is integrated or episomal . However, the method is restricted by a time-consuming and labor - intensive process as well as a reliance on radiolabeled probes [175-177].

4. Dot-Blot Hybridization

This method is similar to southern blot hybridization, except that it does not include electrophoresis. These tests are suitable for large-scale studies, but is rarely used to its low sensitivity [178].

5. Filtered In Situ Hybridization (FISH)

Cells are filtered onto a membrane where they are lysed and denatured. This test was used in many of the early studies but is now abandoned because it is relatively insensitive and has low specificity and reproducibility [(104)].

6. Hybrid Capture II (HC II)

This method uses viral RNA sequences as probes for viral DNA in clinical samples. The binding between RNA and DNA is stronger than of DNA-DNA [(169)]. Hybrid capture II has been favored by many laboratories because of its simplicity and high sensitivity. A part from immediate typing at the time of cytologic diagnosis, HPV typing with HCII can be performed on archived liquid-based cytology samples for reflex testing [(179)].

2.Materials and methods

This study was carried out at the Department of Pathology, college of Medicine – Kufa University, during the period from December 2010 through June 2011. All the cases included in this study were collected from the major teaching hospital and some private laboratories in Al- Najaf.

2.1.Selection of cases

a) Study group: Forty three cases with carcinoma of the uterine cervix were included in this study. These patients had hysterectomy and their ages were ranging from 28 to 65 years. All cases were confirmed by the review of prepared Haematoxylin and Eosin stained slides by certified pathologists. These cases were

collected from laboratory of Histopathology in Alsader Teaching Hospital in Al-Najaf and from four private laboratories in middle of Iraq.

b) Control group: Thirty two cases of normal looking cervical tissue from patients presented with cervical biopsy (manshaister operation) and hysterectomized patients for other gynecological causes were selected and regarded as a control group of the same age group. Each step was done for the control group in parallel with the study group.

c) Positive control: Parallel positive control sections were processed with each set of immunohistochemical and chromogenic in situ hybridization staining . Positive controls of carcinoma of cervix tissue sections, which are known to express HPVs, were used with each run.

d) Negative controls: Sections untreated with the reactive components (the probe in CISH technique and primary antibody in IHC technique) were considered negative controls for each set of slides.

2.2.Immunohistochemistry

Formalin-fixed paraffin-embedded blocks of each biopsy were subjected to cut as serial thin section of (4) thickness and were sticked on charge slides.

2.2.1. Equipments and materials

A-The following equipments and materials were used throughout

the work of research:

1. Water bath, drying oven capable of maintaining 60 ^{o}C or less, humidity chamber and hot plate.
2. Automatic micropipettes of different capacities with tips, pastures pipettes, and eppendorf tubes.
3. Timer with alarm, gloves, cotton swabs, and tissue papers.
4. Binocular Light microscopy.
5. Positively charged slides, cover slides, and slide holders.
6. Staining jars of different sizes and callipered cylinders.
7. Pap pen.
8. Xylene, hematoxylin stain, Distilled water, Ethanol of different concentrations, and DPX..

B. Antibody Diluent: Antibody Diluent with Background Reducing Components, 125ml, Code S3022, LOT 00002288, Dako North America, Inc. 6392 Via Real Carpinteria, CA 93013 USA was used for HPV in the dilution range of 1:50 .

C. **Antigen retrieval:** Target Retrieval Solution, 500 ml, PH 9, Ready-To-Use, Code S2368, LOT 00026677, Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark .

D. **Buffer solution:** Tris buffered Saline with Tween 20.10× Concentrate Code S3306 used for HPVs. DakoCytomation Denmark A/S Produktionsvej 42 DK-2600 Glostrup Denmark. Dako, Inc. 6392 Via Real Carpinteria, CA 93013 USA.

E. Staining kit

DakoCytomation, Ready-To-Use staining kit, Code K0679, sufficient for 150 tissue sections, based on 100 micro liter per section, which includes the following materials:

1- Peroxidase Block, 1x15 ml, 3% hydrogen peroxide in water.

2- Biotinylated Link, 1x15 ml, biotin labeled affinity isolated goat anti-rabbit and goat anti-mouse immunoglobulins in phosphate buffered saline (PBS), containing stabilizing protein and 0.015 mol/L sodium azide.

3- Streptovidin-HRP: 1x15 ml, Streptovidin conjugated to horseradish peroxidase in PBS containing stabilizing protein and anti-microbial agents.

4- DAB substrate buffer, 1x18 ml, Imidazole-HCL buffer PH 7.5 containing hydrogen peroxide and an anti-microbial agents.

5- DAB chromogen, 1x1 ml, 3,3’-diaminobenzidine in chromogen solution.

6- Accessories: calibrated test tube, plastic Pasteur pipette.

2.2.2. Immunohistochemical staining protocol (Technique)

The immunostaining method used in the current study was Labelled Strept-Avidin Biotin ($LSAB^{+}$) technique which was applied for HPVs staining and included the followings[(143)]:

Tissues: 4 µm sections of multi-block with 10 % Neutral Buffered Formalin fixed and paraffin embedded human tissue. Mounted on Silanized slides (S3003). The sections were dried for 60 minutes at 60°C.

Primary antibody: Antibodies are diluted in Dako Antibody Diluent (S3022).

Control: The negative control is pretreated and then incubated in Antibody Diluent step in the protocol.

Deparaffinization: This has been performed previously by immersion in the followings:

1. Two changes of xylene for 10 minutes each.
2. Absolute ethanol for 3 minutes.
3. 95% ethanol for 3 minutes.
4. 70% ethanol for 3 minutes
5. 30% ethanol for 3 minutes
6. Immersion in deionized water for 3 minutes.

Pretreatments:[Heat induced epitope retrieval in microwave-oven (MWO)] **1.** 250 ml heating fluid,10/1 mM Target Retrieval Solution, PH 9, is poured into a plastic container. The slides are placed in a plastic slide holder and transferred to

the plastic container, the slide holder are fill up with slides, so the number of slides is the same each run. The lid is put on and the plastic container is placed in the MWO. At each run, the same numbers of containers are heated, containers without slides must contain 250 ml distilled water.

2. Set the MWO at maximum effect and heat till the fluid boils.
3. Set the MWO at mid effect (approximately 350W) and heat for 15 minutes.
4. Take out the containers from the MWO and let the slides rest in the hot fluid for 20 minutes.

5.Place the containers under gentle rinse water for 5 minutes.
6. Proceed with the immunoprotocol as the following:

- Wash in Tris Buffered Saline (TBS) (S3006) for 5 minutes.
- Encircle tissue with Pap Pen .Wipe off buffer 1/2 cm above and below the tissue and draw a line with the Pap Pen.
- Wash in TBS for 5 minutes.
- Incubate with Peroxidase Blocking-Reagent (K0679) for 10 minutes[1].
- Wash in TBS for 5 minutes.
- Incubate in Primary Antibody[1] for 30 minutes.
- Wash in TBS for 2×5 minutes.
- Incubate with Biotinylated Link Antibody (K0679)[1] for 15 minutes.
- Wash in TBS for 2×5 minutes.
- Incubate with Streptavidin/Peroxidase (K0679)[1]for 15 minutes.

[1]Wipe off TBS and dry the slide outside the Pap-circle and apply 3 - 5 drops of the reagent.

●Wash in TBS for 2×5 minutes.

●Incubate with DAB+ (K0679)*[2] for 10 minutes.

●Wash in TBS for 2 minutes.

●Wash in distilled water for 2 minutes.

●Counterstain in Mayer's Hematoxylin (S3309) for 2 minutes.

●Wash in rinse water for 5 minutes.

●Mount with Faramount (S3025) or dehydrate and cover slide putes (Note : The slides must not dry out during the whole procedure).

2.3.In Situ Hybridization for HPV DNA detection

Formalin-fixed paraffin-embedded blocks of each biopsy were subjected to cut as serial thin section of (4) thickness and were sticked on charge slides.

2.3.1. Equipments and materials

A- The following equipments and materials were used throughout the work of research:

1. Water bath, drying oven capable of maintaining 60 ^{o}C or less, humidity chamber and hot plate.
2. Automatic micropipettes of different capacities with tips, pastures pipettes, and eppendorf tubes.

[2] 1 mL of DAB substrate buffer is transferred to a small test tube and 1 drop of DAB chromogen is added and mixed.

3. Timer with alarm, gloves, cotton swabs, and tissue papers.
4. Binocular Light microscopy.
5. Positively charged slides, cover slides, and slide holders.
6. Staining jars of different sizes and callipered cylinders.
7. Hot air oven (Gallenkamp oven BS, England)
8. Xylene, hematoxylin stain, Distilled water, Ethanol of different concentrations, and DPX.
9. Incubator (Memert, Germany).
10. Forceps, Thermometer, Graduated cylinders.

B. Tris-Buffer saline/Tween(TBST): Tris buffer solution for Genpiont and wide spectrum DNA probes dilute the TBST concentrate (code S3306) 1:10 in deionized or distilled water.

C. Pepsin : It is used for the proteolytic digestion of paraffin-embedded, formalin-fixed tissues prior to DNA in situ hybridization procedures. The entire contents of one packet is dissolved in 250 mL of 0.2 N HCl.

D. 0.3% H2O2 in methanol: BDH Chemical Ltd; England.

E. Staining Kit

DakoCytomation, Ready-To-Use staining kit, Code K0620, sufficient for 65 tissue sections, based on 150 micro liter per section, which includes the following materials:

1. Stringent Wash Solution: Dilute the stringent wash concentrate 1:50 in deionized or distilled water and heat to the appropriate temperature in a water bath.

2.Primary streptavidin-HRP concentrate: 1x0.2 ml, Streptovidin conjugated to horseradish peroxidase in PBS containing stabilizing protein and anti-microbial agents, should be diluted 1:100 in the Primary Streptavidin-HRP Diluent provided prior to use.

3.Primary streptavidin-HRP Diluent: 1x15 ml**,** diluted the primary streptavidin-HRP 30 minutes prior to use.

4.DAB substrate buffer: 1x10 ml, Imidazole-HCL buffer PH 7.5 containing hydrogen peroxide and an anti-microbial agents.

5. DAB chromogen concentrate: 1x0.2 ml, 3,3'-diaminobenzidine dilute 1:50 in DAB Substrate Buffer immediately before use.

6. Biotinyl tyramide solution 1x10 ml (amplification reagent).

7.Secondary Streptavidin-HRP solution: 1x10ml**,** Streptovidin conjugated to horseradish peroxidase in PBS containing stabilizing protein and anti-microbial agents.

2.3.2.CISH staining protocol

Tyramide Signal Amplification (TSA) detection system used on specimens of four microns thick sections were cut from the formalin-fixed, paraffin-embedded blocks and placed on positive charged slides.

- **Deparaffinization** by heating the slides in an oven at 60°C for 60 minutes.

1. Two changes of xylene for 10 minutes each.
2. Absolute ethanol for 3 minutes.
3. 95% ethanol for 3 minutes.
4. 70% ethanol for 3 minutes
5. 30% ethanol for 3 minutes
6. Immersion in deionized water for 3 minutes

- **Sampling pretreatment:** It requires pretreatment to provide the hybridization probe access to target nucleic acid sequences and can be performed by the following:

❖ Incubated sections in 0.8% pepsin (dissolved packed in 250 ml 0.2N HCL) at 37 C for (5-10 minutes).

❖ Rinse the section in two change of deionised water for 2 minutes each.

❖ Immersed in 0.3% H2O2 in methanol for 20 Minutes.

❖ Rinse the sections in two change of deionised water for 2 minutes each.

- **Hybridization and Stringent Wash**

➢ The sections allow air dry briefly, add drop of biotinylated DNA probe and cover slip is applied.

➢ Placing on flat block surface at 92C for 5 min, Transferred to pre-warmed humid chamber for hybridization at 37C (12 hr.).

- Cover slip is remove by immerse in room temperature in TBST 1X, then slides transferred to fresh TBST bath for 2 minutes before stringent washing at 48 °C for 30 minutes.
- Rinsing the slides in two changes of 10X TBST for 2 minutes each.

- **Staining Procedure**

- Add 3-4 drops per slide of primary Strep-Avidin~HRP using a 1:100 dilution. The antibody is dissolved in 0.5% blocking solution, slides are incubated in humidity chamber for 15 minutes at RT.
- Rinsing the slides in two changes of 10X TBST for 2 minutes each.
- Shake off excess buffer, apply 3-4 drops of tyramide solution, incubate slides in humidity chamber for 15 min at RT.
- Rinsing the slides in two changes of 10X TBST for 2 minutes each.
- Shake off excess buffer, add 3-4 drops per slide of secondary Strep-Avidin~HRP, incubate slides in humidity chamber for 15 minutes at RT.
- Apply substrate-chromagen solution (DAB) for 5-15 minutes.
- Wash in distilled water.
- Counter stain with Meyer‹s hematoxyllin and mounting .

2.4.Tumor markers used

1-Anti-HPV monoclonal antibody.

2- Wide Spectrum HPV probe.

3- GenPoint™ HPV DNA Probe**.**

2.5.1. Anti-Human Papillomavirus (HPV): Monoclonal mouse anti-human (HPV) protein, 0.2 ml, clone K1H8, code M3528, Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark was used as primary antibody for the detection of HPV protein.

2.5.2.Wide Spectrum HPV probe: Biotinylated DNA Probe, 1 mL of in situ hybridization solution, Code Y1404, Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark was used in in situ hybridization staining procedure using Dako GenPoint™ Detection System (code K0620) for the detection HPV-positive specimens for the following HPV types: 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52.

2.5.3. GenPoint™ HPV DNA Probe: is an HPV probe cocktail, 1 mL of in situ hybridization solution, Code Y1443, Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup, Denmark was used in in situ hybridization staining procedure using Dako GenPoint™ Detection System (code K0620) for the detection High risk group of HPV-positive specimens for High risk group of HPV.

2.5. Scoring system

The criterion for positive immunoreaction is dark brown precipitate at in the nucleus for HPV. The intensity of the staining was assessed by counting the percentage of positive cells in 100 malignant cells at objective 40 total magnification. The immunostaining was calculated as the percentage of immunoreactive cells per total number of malignant cells. Each sample was scanned for at least five fields randomly with a high power magnification[180-185].

Scoring system: based on the positive nuclear staining fraction of the tumor cells[186]

Score 0 = no staining

Score +1= 1%-10% of tumor cells.

Score +2= 11%-25% of tumor cells.

Score +3= 26%-50% of tumor cells.

Score +4= 51%-100% of tumor cells.

Statistical analyses

Statistical analyses of all results were performed by the help of SPSS software statistical package (version 15) using Chi Square test and P value at level of significance less than 0.05.), and correlation- Regression test (R at a significant level of 0.3).

Study population

Our retrospective study group consisted of 75 samples archival cervical tissue. Blocks had been fixed with formalin and embedded with paraffin wax. Ages of patients ranges from 28 to 65 years, with a mean of (46.02 ± 7.690 S.D. years). These cases were collected from laboratory of Histopathology in Alsader Teaching Hospital in Al-Najaf and from four private laboratories in this governorate. These patients either had hysterectomy or punch and cone biopsy. Ethical approval for use of all specimens was obtained and the histopathology diagnosis was confirmed by review of freshly prepared hematoxylin and eosin-stained slides and classified according to criteria outlined by the World Health Organization (WHO). The histological diagnosis of these tissue blocks was normal in 32 cases and 43 with invasive cervical carcinoma (squamous cell carcinoma in 40 cases and adenocarcinoma in 3 cases). Details of the subjects and their numbers and range of age are shown in Table (3.1).

Table 3.1: The characteristic features of the presented patients with malignant cervical tumors.

Parameter	NO.	percentage	Total
Age			
21-30	2	4.65%	
31-40	6	13.95%	
41-50	25	58.14%	43
51-60	7	16.28%	
61-70	3	6.98%	
Histological type			
Squamous cell carcinoma	40	93%	43
Adenocarcinoma	3	7%	
Grade			
Well Differentiated	29	67.4%	
Moderately Differentiated	11	25.6%	43
Poorly Differentiated	3	7%	
Stage(T)			
Ta	6	13.9%	43
T1	12	27.9%	
T2	10	23.3%	
T3	8	18.6%	

T4	7	16.3%	

3.1. Clinicopathological analysis

Forty-three cases of cervical cell carcinoma were included in this study. Clinicopathological assessment revealed that 40 (93%) patients were with squamous cell carcinoma and 3 (7%) were with adenocarcinoma (Figure3.1).

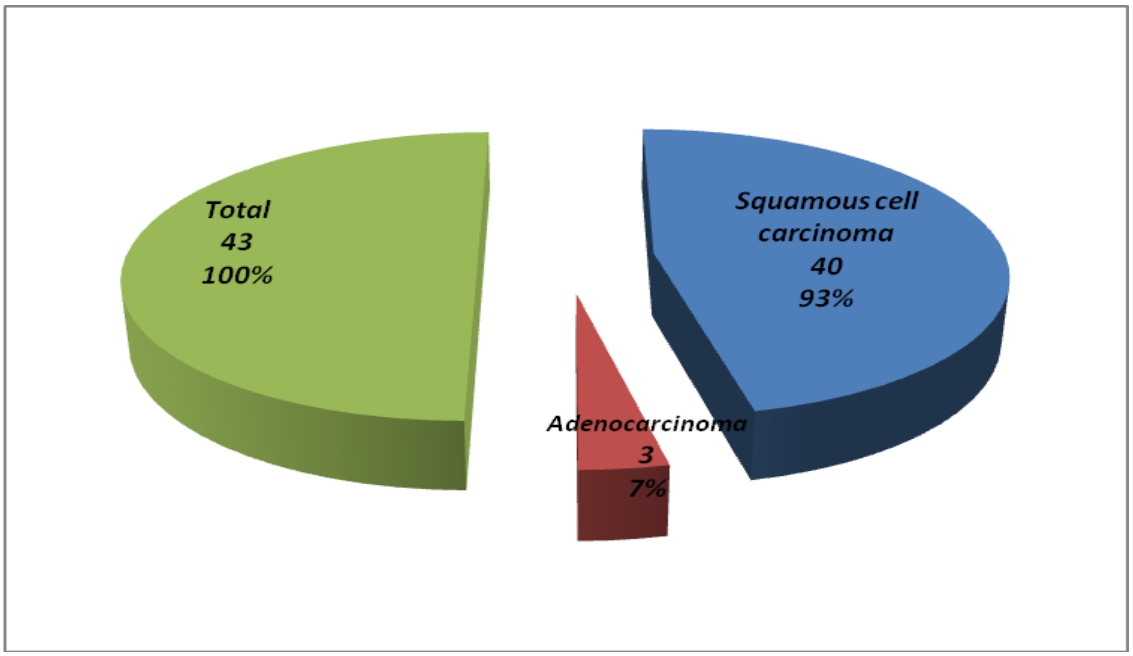

Figure 3.1: Histopathological types of the presented cervical carcinoma patients.

Assessment of age presentation of the patients revealed that 2 (4.65%) patients were seen in age group (21-30 years), 6 (13.95%) were seen in age group

(31-40 years), 25(58.14%) in age group (41-50 years), 7 (16.28%) in age group (51-60 years), and 3 (6.98%) in age group (61-70 years) . The age of patients ranged between 28-65 years with a Mean=46.02±7.960 (Mean ± SD) years. 2 patients (4.65%) were within the 2nd decade of life and more , while 6 patients (13.95%) were within the 3th decade , 25 cases (58.14%) within the 4th decade,7 patients (16.28%) within 5th decade and only 3 case (6.98%) was within the 6th decade as shown in (Table3.2) (Figure 3.2).

Table 3.2: Age distribution of the presented cervical cell carcinoma patients.

Age group	NO.	MEAN	Std.Deviation
21-30	2	28.5	0.707
31-40	6	37.17	2.483
41-50	25	45.28	2.670
51-60	7	54.14	3.132

61-70	**3**	**62.67**	**2.082**
Total	**43**	**46.02**	**7.960**

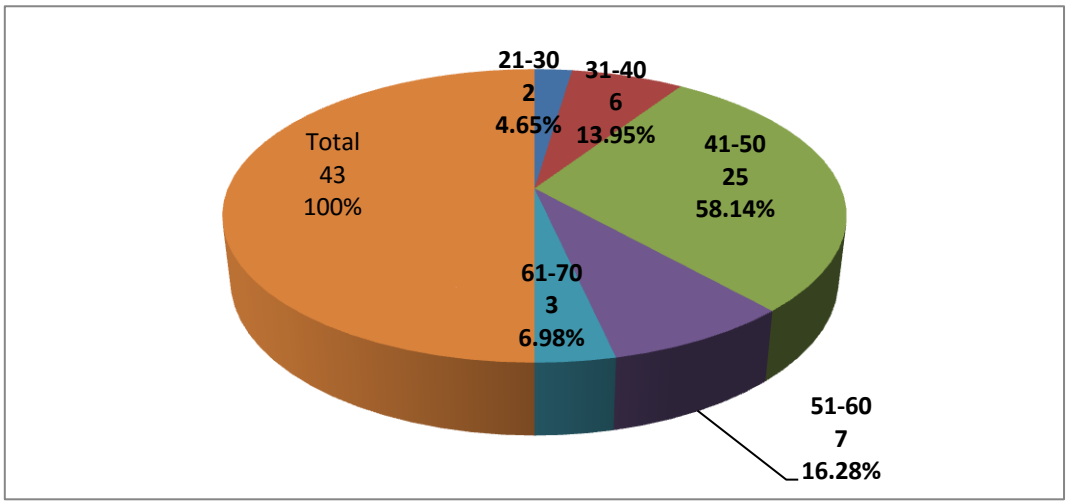

Figure 3.2: Age distribution of the presented cervical carcinoma patients.

Grading of the presented malignant cases were assessed according to the WHO grading system of cervical carcinoma , revealing that grade I was reported in 29 (67.44%) cases, grade II in 11(25.58%), while those of grade III were 3 (6.98%) cases (Figure 3.3) .

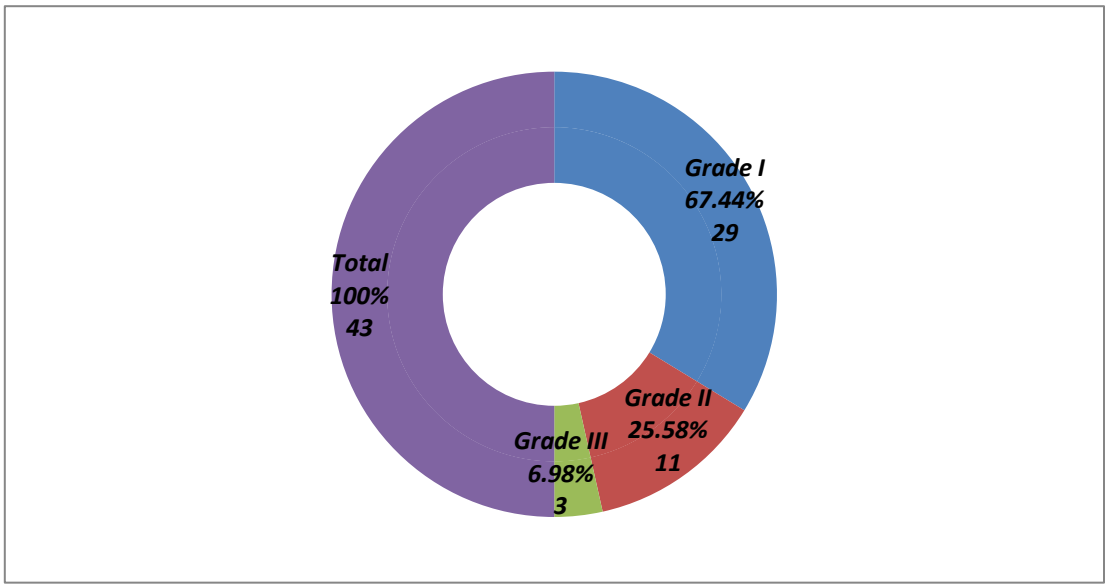

Figure 3.3: The percentage of grades of the presented cervical carcinoma patients.

Assessment of the size (T) of the tumor of presented cervical cell carcinoma according to the TNM staging system revealed that : 6 (13.95%) cases were of Ta, 12 (27.91%) of T1, 10 (23.26%) of T2, 8 (18.60%) of T3, while T4 formed 7 cases (16.28%) (Table3.1) (Figure 3.4).

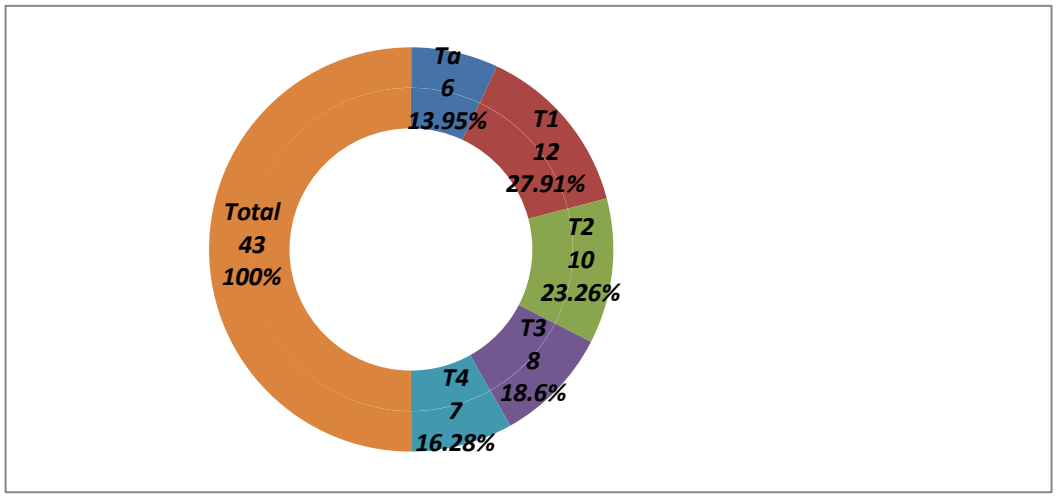

Figure 3.4: The frequency of the tumor sizes (T) of the presented cervical carcinoma patients.

3.2. HPV Immunohistochemical study

3.2.1. HPV immunoexpression in benign and malignant cervical tissue

The results revealed that immunostaining of HPV protein was exclusively accumulated in the nucleus (nuclear stain) of malignant cells.

In all sections of benign control cervix tissues (mature squamous epithelium), none of them revealed a positive immunoexpression for HPV in the nucleus . In the studied group, HPV immunoexpression was reported in 18 out of 43 cases of cervical carcinoma represented (41.9%) within type of tissue. 25 cases were negative represented (58.1%) within type of tissue , with significant difference in comparison with control group ($P < 0.001$) (Table 3.3) (Figure 3.5 and Figure 3.6).

HPV immunoreactive cells were reported in 45% (18 out of 40) of cases with squamous cell carcinoma , while none (0 out of 3) of cases with adenocarcinoma was found to express HPV in their nuclei with a significant difference among histological types of tumor ($P<0.05$) (Table 3.4).

3.2.2. HPV immunoexpression and age of patient

HPV immunoexpression in relation to age distribution of patients revealed that the HPV immunoexpression was detected in 2 out 2 cases in age group 21-30 years represented (100%) within age group of patients. 3 out 6 cases in age group 31-40 years represented (50%) within age group of patients.12 out 25 cases in age group 41-50 represented (48%) within age group of patients. The HPV immunostaining was 14.3% (1out 7) in age group 51-60 years. The difference

among these age groups were statistically no significant (P>0.05). However, the immunodetection rate of HPV looks positively well correlated to the age of patient (r =0.392) (Table3.5) .

3.2.3.HPV immunoexpression and grade of tumor

HPV immunohistochemical analysis in relation to grade of tumor revealed that 14 out of 29 of grade I was positive represented (48.3%) within grade of tumor. 3 out of 11 cases of grade II were positive represented (27.3%) within grade of tumor. 1 out of 3 cases of grade III were positive represented (33.3%) within grade of tumor and. There was no significant difference among these grades (P value >0.05) (Table 3.6) .

The nuclear staining intensity of HPV immunoexpression was assessed in correlation to the grade of tumor. Score +1 was reported in 3 out of 43 cases; 2 grade I and one grade II. Score+2 was found in 4 cases; 3 in grade I and one in grade II. Score +3 was reported in 4 cases of cervical carcinoma ; all of which were grade I. Score +4 was reported in 7 case of cervical carcinoma; 5 in grade I, one in grade II and one in grade III (Table 3.24) (Figure 3.7).

There was no significant correlation between intensity of HPV immuno-expression and the grades of cervical carcinoma (P>0.05). A high proportion of HPV immunoexpression was reported among those with well differentiated cervical carcinoma , while less proportion of HPV was reported among those with poorly or moderately differentiated cervical carcinoma .

3.2.4.HPV immunoexpression and size (T) of tumor

HPV immunoexpression was reported in 2 out of 6 cases of Ta represented (33.3%) within size (T) of tumor. 7 out of 12 cases of T1 represented (58.3%) within size (T) of tumor. 3 out of 10 cases of T2 represented (30%) within size (T) of tumor. 2 out of 8 cases of T3 represented (25%) within size (T) of tumor. 4 out of 7 of T4 represented (57.1%) within size (T) of tumor. There was no significant difference between different tumor sizes (P >0.05) (Table 3.7) .

The nuclear staining intensity of HPV overexpression was assessed in relation to the stage (T) of tumor .In stage (Ta) cervical carcinoma , one case was found to have score +1 and another with score +2. In stage (T1) cervical carcinoma, one case was found to have score +1, 2 cases score +2 , 2 cases score +3, and 2 cases score +4. In stage (T2) cervical carcinoma. one case was found to have score +3, and 2 cases score +4. In stage (T3) cervical carcinoma , one case was found to have score +3, and another with score +4. In cases with stage (T4), there were two cases distributed as score +1, score +2, and two cases with score +4. Score +4 nuclear intensity was reported in 7 cases of cervical carcinoma; two cases with stage (T1), (T2) and (T4) , and only one case with stage (T3) . Score +3 nuclear intensity was reported in 4 cases of cervical carcinoma; two cases with stage (T1) , one case with stage (T2) and one of stage (T3). Score +2 nuclear intensity was reported in 4 cases of cervical carcinoma; two cases with stage (T1), one case with stage (Ta) and one with stage (T4). Score +1 nuclear intensity was reported in 3 cases of cervical carcinoma; one case with stage (Ta), one case with stage (T1) and one with stage (T4).There was no significant difference between different tumor sizes (P value > 0.05) (Table 3.24).

The immunostaining character and localization of HPV in cervical carcinoma in our study is shown in the following figures :

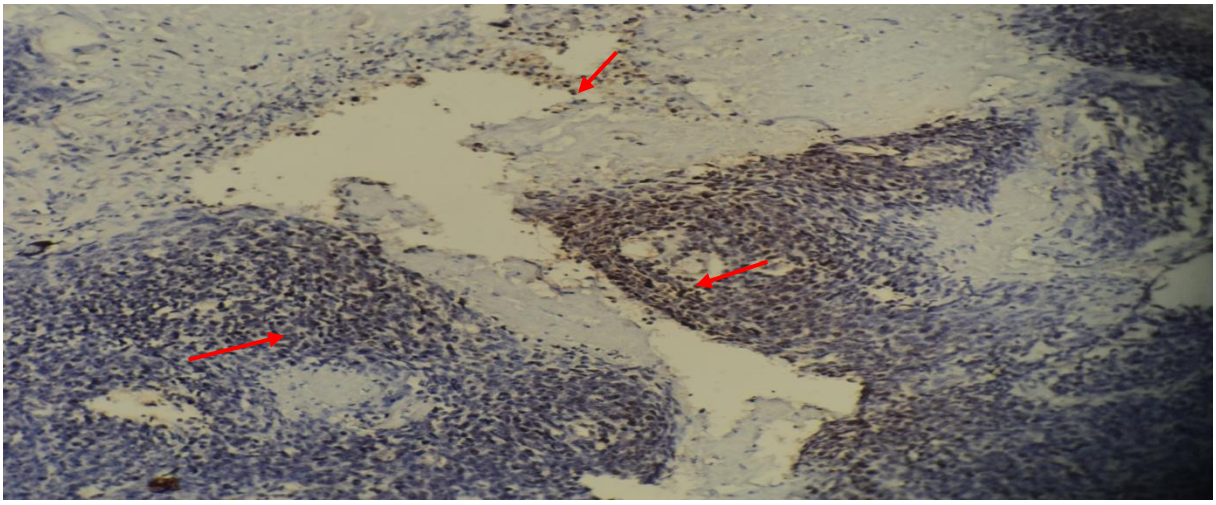

Figure 3.5: HPV immunohistochemical nuclear staining pattern score+2(⟶) in cervical tissues involved by Cervical carcinoma (X10).

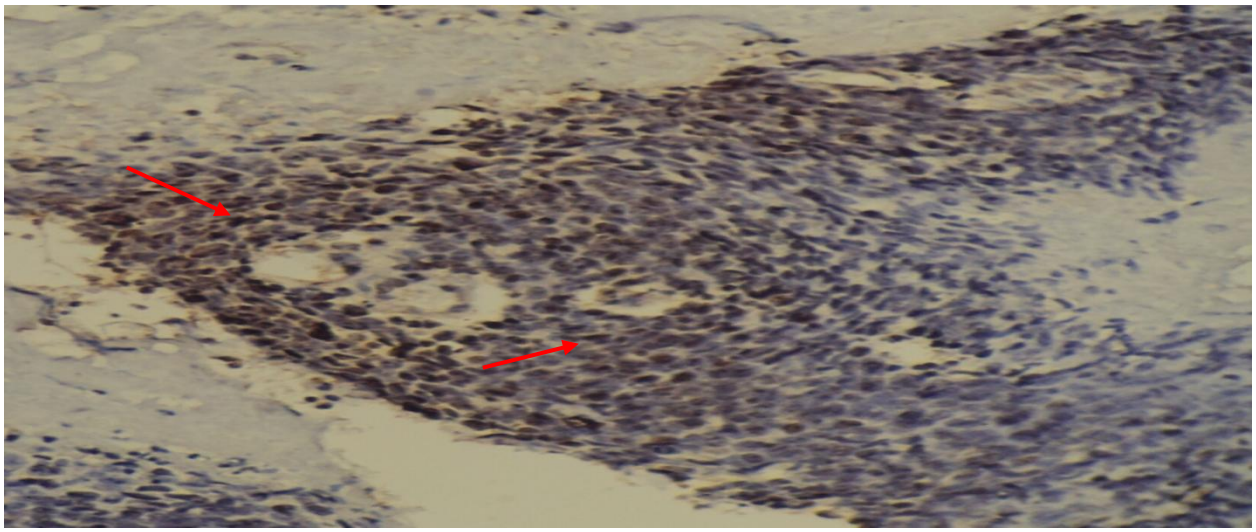

Figure 3.6: A higher power of figure 3.5 to show in more detail the localization of HPV protein in the nucleus, nuclear staining pattern, score+2 (⟶) (X40).

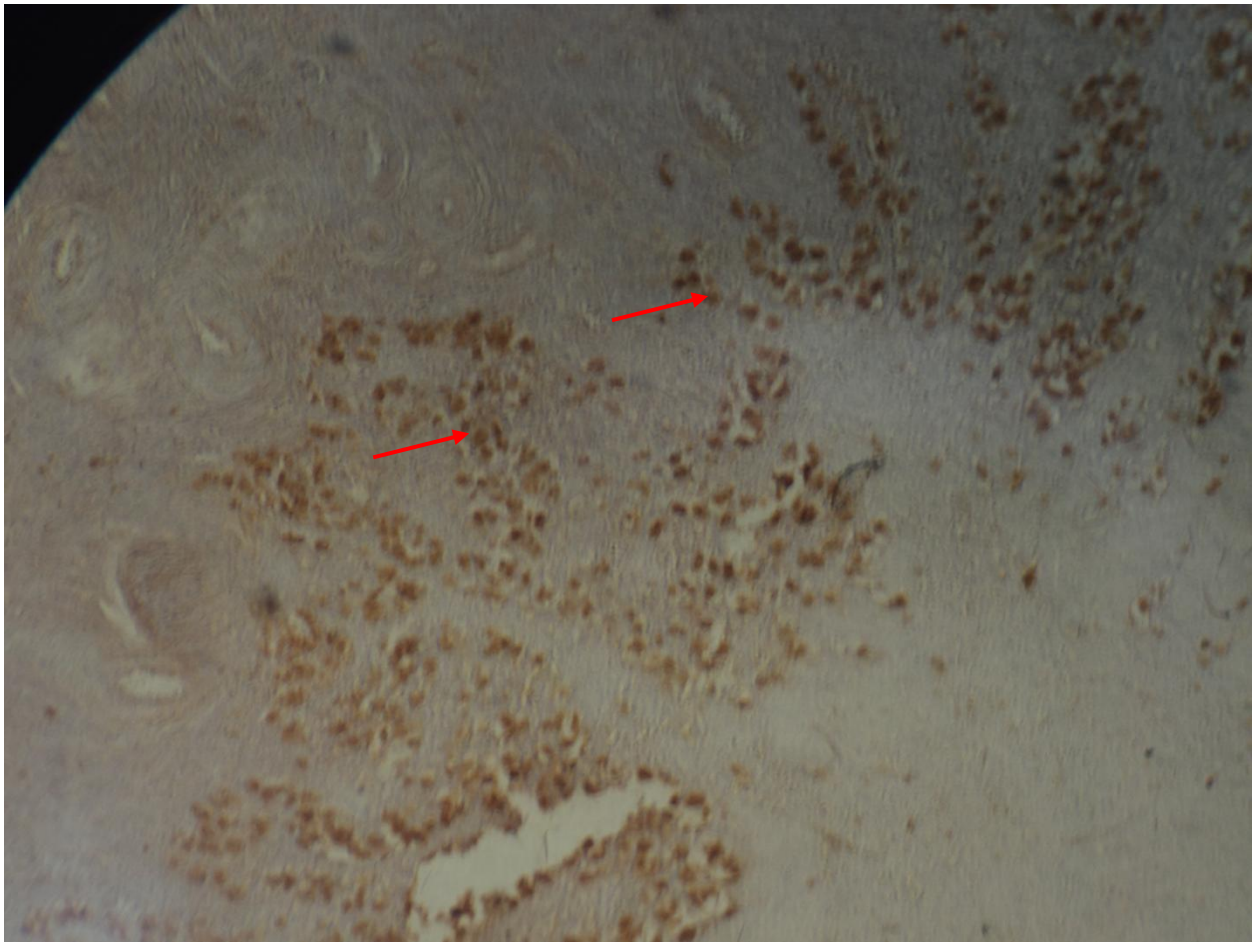

Figure 3.7: Cervical carcinoma show strong score +3 (⟶) immunohistochemical nuclear stained of HPV (X10).

Table 3.3:HPV immunoexpression in cervical carcinoma in comparison to normal cervical tissue.

Type of tissue		HPV- Immunostaining		Total
		Positive	Negative	
Benign	Count	0	32	32
	%within tissue type	0%	100%	100%
Malignant	Count	18	25	43
	%within tissue type	41.9%	58.1%	100%
Total	Count	18	57	75
	% within tissue type	24%	76%	100%

(P<0.0001 , r=0.485)

Table 3.4:HPV immunoexpression in different histological types of cervical carcinoma.

Histological type		HPV immunostaining		Total
		Positive	Negative	
Squamous cell carcinoma	Count	18	22	40
	%within histological type	45%	55%	93%
Adenocarcinoma	Count	0	3	3
	%within histological type	0%	100%	7%
Total	Count	18	25	43
	%within histological type	41.9%	58.1%	100%

(P<0.05 , r>0.3)

Table 3.5:The relation of HPV immunodetection and age of the patients.

Age group		HPV-immunostaining		Total
		Positive	Negative	
21-30	Count	2	0	2
	%within age group	100%	0%	100%

31-40	Count %within age group	3 50%	3 50%	6 100%
41-50	Count %within age group	12 48%	13 52%	25 100%
51-60	Count %within age group	1 14.3%	6 85.7%	7 100%
61-70	Count %within age group	0 0%	3 100%	3 100%
Total	Count %within age group	18 41.9%	25 58.1%	43 100%

(P<0.05 , r=0.392)

Table 3.6: The relation of HPV immunodetection and grade of tumor.

Grade of tumor		HPV-immunostaining		Total
		Positive	Negative	
Grade I	Count	14	15	29
	%within tumor grade	48.3%	51.7%	100%
Grade II	Count	3	8	11
	%within tumor grade	27.3%	72.7%	100%
Grade III	Count	1	2	3
	%within tumor grade	33.3%	66.7%	100%
Total	Count	18	25	43
	%within tumor grade	41.9%	58.1%	100%

(P>0.05 , r=0.162)

Table 3.7: The relation of HPV immunodetection and size(T) of tumor.

Stage(T) of tumor		HPV-immunostaining		Total
		Positive	Negative	
Ta	Count	2	4	6
	%within tumor stage(T)	33.3%	66.7%	100%
T1	Count	7	5	12
	%within tumor stage(T)	58.3%	41.7%	100%
T2	Count	3	7	10
	%within tumor stage(T)	30%	70%	100%
T3	Count	2	6	8
	%within tumor stage(T)	25%	75%	100%
T4	Count	4	3	7
	%within tumor stage(T)	57.1%	42.9%	100%
Total	Count	18	25	43
	%within tumor stage(T)	41.9%	58.1%	100%

(P>0.5 , r=0.006)

3.3. HPV chromogenic in situ hybridization (CISH) study

3.3.1.GenPoint™ HPV amplification in benign tissue and cervical carcinoma

The results revealed that CISH staining of HPV DNA was exclusively accumulated in the nuclei (nuclear stain) of malignant cells.

In all sections of benign control cervical tissues (mature squamous epithelium), none of them revealed a positive amplification for HPV in the nucleus . In the studied group, however, HPV amplification was reported in 17 out of 43 cases of cervical carcinoma represented (39.5%) within type of tissue, and 26 cases were negative represented (60.5%) within type of tissue. There was a positive correlation between HPV amplification and the types of tissue (r = 0.467), with a significant difference among tissue types (P<0.0001) (Table 3.8) (Figure 3.8 and Figure 3.9).

HPV GenPoint™ amplification cells were reported in 42.5% (17 out of 40) of cases with squamous cell carcinoma , while none (0 out of 3) of cases with adenocarcinoma was found to express HPV in their nuclei with a significant difference among histological types of tumor (P<0.05) (Table 3.10).

3.3.2. Wide Spectrum HPV amplification in benign tissue and cervical carcinoma

The results revealed that CISH staining of HPV DNA was exclusively accumulated in the nuclei (nuclear stain) of malignant cells.

In all sections of benign control cervical tissues (mature squamous epithelium), none of them revealed a positive amplification for HPV in the nucleus. Contrarily, in the studied group, HPV amplification was reported in 15 (34.9%) out of 43 cases of cervical carcinoma represented (34.9%) within type of tissue. And 28(65.1%) cases were negative represented (65.1%) within type of tissue. There was a positive correlation between HPV amplification and the types of tissue ($r = 0.431$), with a significant difference among tissue types ($P<0.0001$) (Table 3.9) (Figure 3.10 and Figure 3.11) .

HPV wide spectrum amplification cells were reported in 37.5% (15 out of 40) of cases with squamous cell carcinoma , while none (0 out of 3) of cases with adenocarcinoma was found to express HPV in their nuclei with a significant difference among histological types of tumor ($P<0.05$) (Table 3.11).

3.3.3. GenPoint™ HPV amplification and age of patient

Detection rate of HPV ISH in relation to age distribution of patients revealed that HPV DNA (GenPoint™) was detected in one out 2 cases in age group 21-30 years represented (50%) within age group of patients. 3 out 6 cases in age group 31-40 years represented (50%) within age group of patients. 11 out 25 cases in age group 41-50 represented (44.0%) within age group of patients. Positive CISH detection rate for HPV was 28.6% (2out 7) in age group 51-60 years. There was no significant difference among age of patients ($P>0.05$)($r=0.228$) (Table 3.12) (Figure 3.12).

3.3.4. Wide Spectrum HPV amplification and age of patients

Detection rate of HPV DNA ISH in relation to age distribution of patients revealed that HPV DNA Wide spectrum was detected as follow; none of patients in age group 21-30 years were positive CISH detection rate for HPV Wide spectrum, 3 out 6 cases in age group 31-40 years represented (50%) within age group of patients,10 out 25 cases in age group 41-50 represented (40.0%) within age group of patients, while a positive CISH detection rate for HPV Wide spectrum was 28.6% (2out 7) in age group 51-60 years. There was no significant difference among age of patients (P>0.05) (r =0 .114) (Table 3.13) (Figure 3.13) .

3.3.5. GenPoint™ HPV amplification and grade of tumor

Detection rate of chromogenic in situ hybridization (CISH) analysis for HPV (GenPoint™) in relation to grade of tumor revealed that 15 out of 29 of grade I was positive represented(51.7%) within grade of tumor. 2 out of 11 cases of grade II were positive represented (18.2%) within grade of tumor, none cases of grade III were positive. It looks that the detection rate of HPV (GenPoint™) is well correlated to the grade of tumor (r=0.365), without any significant difference among the grade of tumor((P>0.05) (Table 3.14) (Figure 3.14) .

The nuclear staining intensity of HPV CISH was assessed in correlation to the grade of tumor. Score +1 was reported in 3 out of 43 case of cervical carcinoma, all of which were grade I . Score+2 was found 4 cases; 3 grade I and one grade II . Score +3 was reported in 4 cases of cervical carcinoma ; all of which were grade I. Score +4 was reported in 6 case of cervical carcinoma, and 5 in grade I and one in grade II.

The intensity of HPV looks to be well correlated to the grade of tumor , and a high proportion of HPV expression was reported among those with well differentiated cervical carcinoma. Nevertheless, less proportion of HPV was reported among those with poorly or moderate differentiated cervical carcinoma without a significant difference ($P > 0.05$) (Table3.25).

3.3.6. HPV Wide spectrum amplification and grade of tumor

Chromogenic in situ hybridization (CISH) analysis for HPV Wide spectrum in relation to grade of tumor revealed that 13out of 29 of grade I was positive represented (44.8%) within grade of tumor. 2 out of 11 cases of grade II were positive represented (18.2%) within grade of tumor. None cases of grade III were positive. It looks that the detection rate of HPV Wide spectrum is well correlated to the grade of tumor ($r = 0.312$), without significant difference among the grade of tumor ($P>0.05$) (Table 3.15) (Figure 3.15) .

The nuclear staining intensity of HPV CISH was assessed in correlation to the grade of tumor. Score +1 was reported in 2 out of 43 cases of cervical carcinoma, all of which were grade I . Score+2 was found 4 cases; 3 grade I and one grade II . Score +3 was reported in 3 cases of cervical carcinoma ; 3 grade I and one grade II. Nevertheless, score +4 was reported in 5 case of cervical carcinoma; 4 in grade I and one in grade II. The intensity of HPV looks to be well correlated to the grade of tumor , and a high proportion of HPV amplification was reported among those with well differentiated cervical carcinoma. Less proportion of HPV was reported among those with poorly or moderately differentiated cervical carcinoma without a significant difference ($P > 0.05$) (Table 3.26).

3.3.7. HPV (GenPoint™) amplification and size (T) of tumor

HPV (GenPoint™) amplification was reported in 2 (33.3%) out of 6 cases of Ta represented (33.3%) within size (T) of tumor. 4 out of 12 cases of T1 represented (33.3%) within size (T) of tumor. 3 out of 10 cases of T2 represented (30%) within size (T) of tumor. 3 out of 8 cases of T3 represented (37.5%) within size (T) of tumor, and 5 (71.43%) out of 7 cases of T4 represented (71.4%) within size (T) of tumor, without any significant difference among the size (T) of tumor (P>0.05) (Table 3.16) (Figure 3.16) .

The nuclear staining intensity of HPV CISH was assessed in relation to the stage (T) of tumor. In stage (Ta) cervical carcinoma , one case was found to have score +1 and one case score +2. In stage (T1) cervical carcinoma, there were four cases distributed as one case score +1, one case score +2 , one case score +3 and one case score +4, in stage (T2) cervical carcinoma. One case was found to have score +3, and 2 cases score +4, in stage (T3) cervical carcinoma. There were 3 cases distributed as one case score +1, one case score +3, and one case score +4, while in cases with stage (T4) there were five cases distributed as one case score +1, one case score +2, one case score +3 and two cases score +4. Score +4 nuclear intensity was reported in 6 cases of cervical carcinoma; two cases with stage (T2) and (T4) ,one case with stage(T1) and (T3). Score +3 nuclear intensity was reported in 4 cases of cervical carcinoma; one case with stage(T1) , (T2), (T3) and (T4). Score +2 nuclear intensity was reported in 4 cases of cervical carcinoma; one case with stage (Ta), (T1), (T3) and (T4). Score +1 nuclear intensity was reported in 3 cases of cervical carcinoma; one case with stage (Ta), (T1) and (T4). Strong staining pattern was well correlated to the size of tumor. However, there was no significant difference between different tumor sizes (P value > 0.05)(Table 3.25).

3.3.8. HPV Wide spectrum amplification and size (T) of tumor

HPV Wide spectrum amplification was reported in 2 out of 6 cases of Ta represented (33.3%) within size (T) of tumor. 3 out of 12 cases of T1 represented (25%) within size (T) of tumor. 3 out of 10 cases of T2 represented (30%) within size (T) of tumor. 3 out of 8 cases of T3 represented (37.5%) within size (T) of tumor, and 4 (57.14%) out of 7 cases of T4 represented (57.1%) within size (T) of tumor, without any significant difference among the size (T) of tumor (P>0.05) (Table 3.17) (Figure3.17).

The nuclear staining intensity of HPV CISH was assessed in relation to the stage (T) of tumor. In stage (Ta) cervical carcinoma , one case was found to have score +1 and one case score +3. In stage (T1) cervical carcinoma, there were three cases distributed as one case score +1, one case score +2, and one case score +4. In stage (T2) cervical carcinoma, there were 3 cases distributed as one case score +2, one case score +3, and one case score +4. In stage (T3) cervical carcinoma , there were 3 cases distributed as one case score +2, one case score +3, and one cases score +4, while in cases with stage (T4) there were four cases distributed as one case score +2, one case score +3 and two cases score +4 . Score +4 nuclear intensity was reported in 5 cases of cervical carcinoma; one case with stage (T1), (T2) and (T3), two cases with stage (T4). Score +3 nuclear intensity was reported in 4 cases of cervical carcinoma; one case with stage(Ta) , (T2), (T3) and (T4). Score +2 nuclear intensity was reported in 4 cases of cervical carcinoma; one case with stage(T1),(T2), (T3) and (T4). Score +1 nuclear intensity was reported in 2 case of cervical carcinoma; one case with stage (Ta), and (T1) . Strong staining

pattern was well correlated to the size of tumor. However, there was no significant difference between different tumor sizes (P value > 0.05)(Table 3.26).

The chromogenic in situ hybridization character and localization of HPV in cervical carcinoma in our study is shown in Figure 3.8-Figure 3.13:

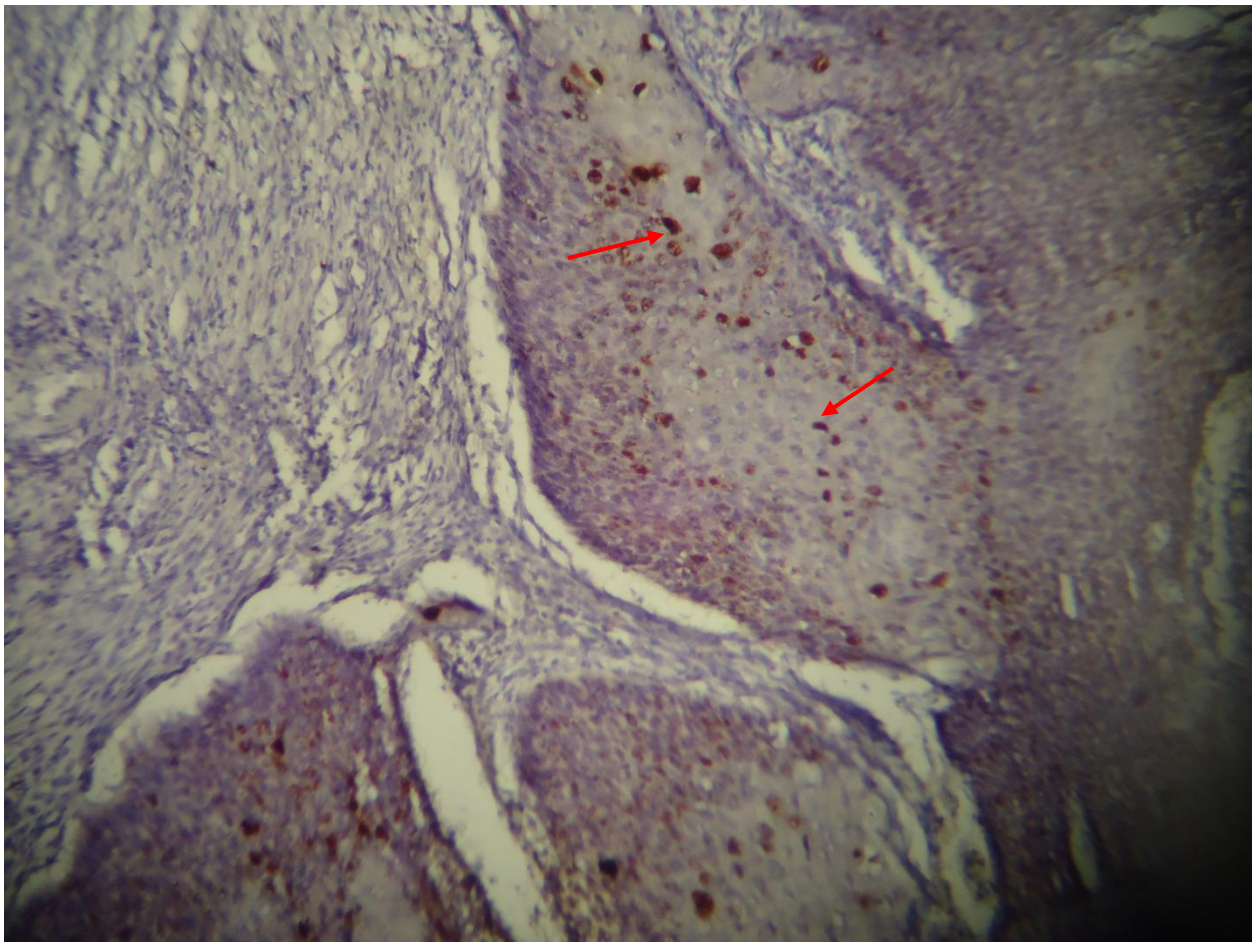

Figure 3.8 : GenPoint™ HPV ISH in cervical tissues involved by Cervical carcinoma, nuclear staining pattern of HPV, score+2 (⟶) (X10).

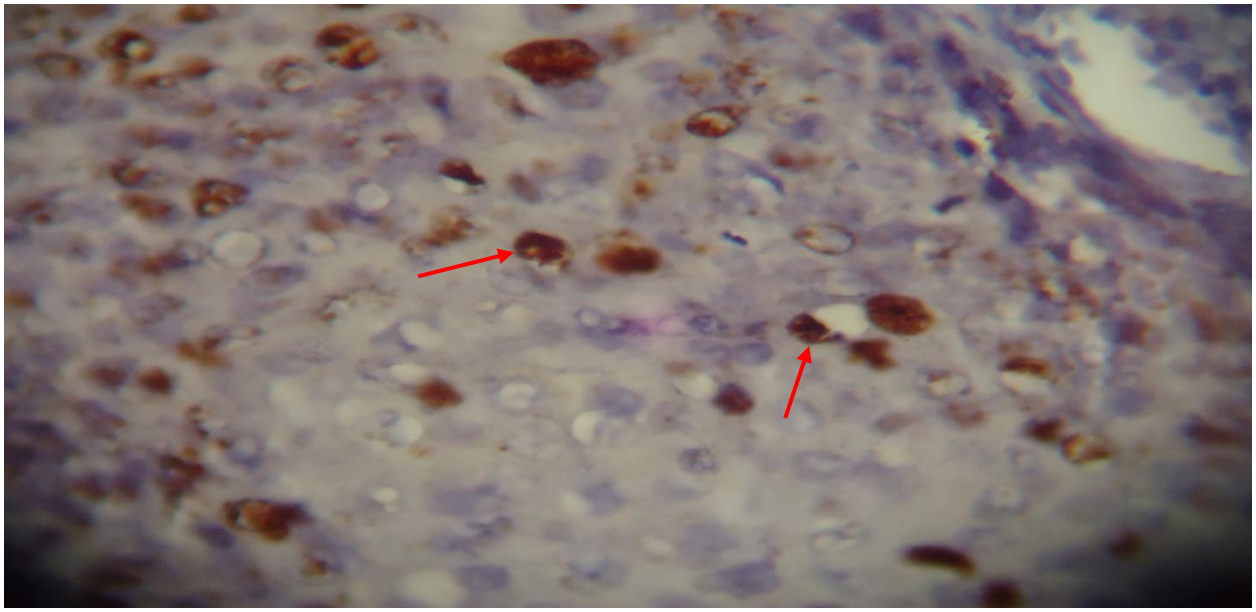

Figure 3.9: A high power of figure 3.8 to show in more detail the localization of HPV DNA in the nucleus , score +2(⟶), nuclear staining pattern of HPV (X40).

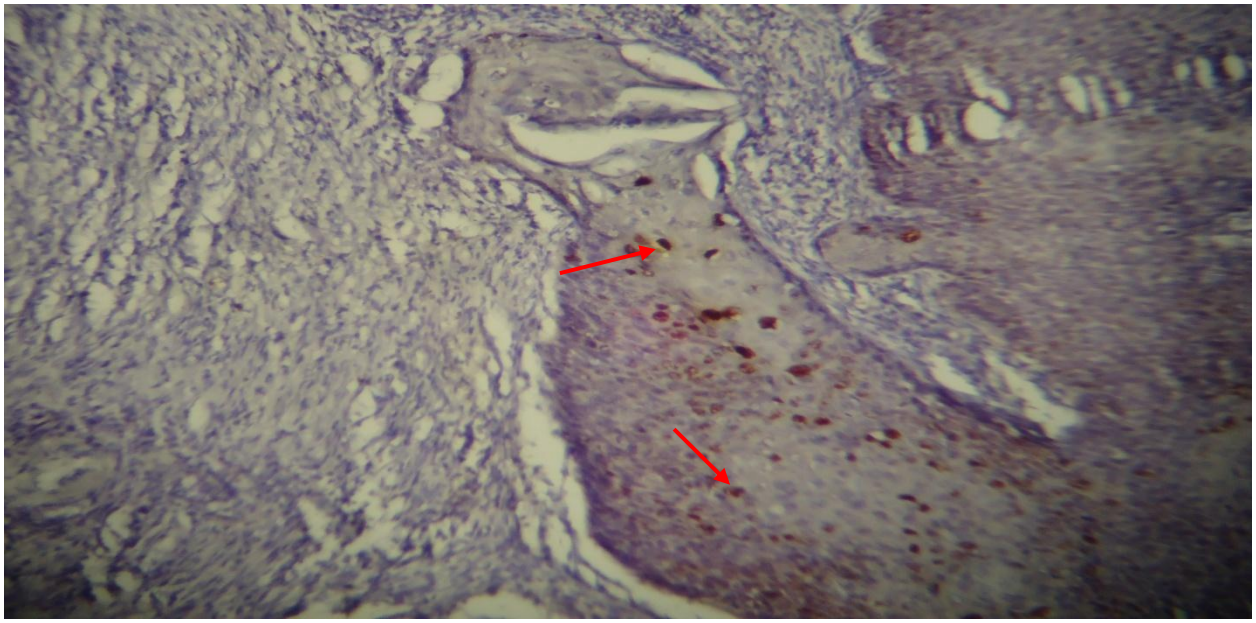

Figure 3.10: Wide Spectrum HPV ISH in cervical tissues involved by Cervical carcinoma, nuclear staining pattern of HPV, score+2 (⟶) (X10).

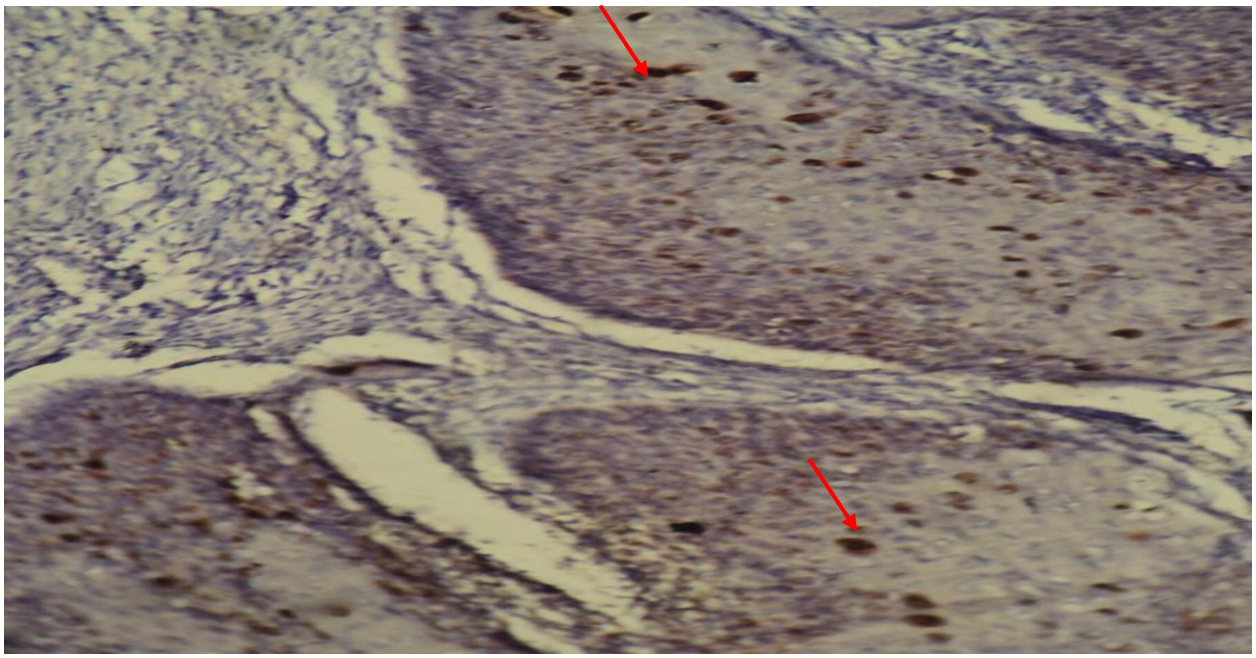

Figure 3.11: A high power of figure 3.10 to show in more detail the localization of HPV DNA in the nucleus , score +2(→), nuclear staining pattern of HPV (X40).

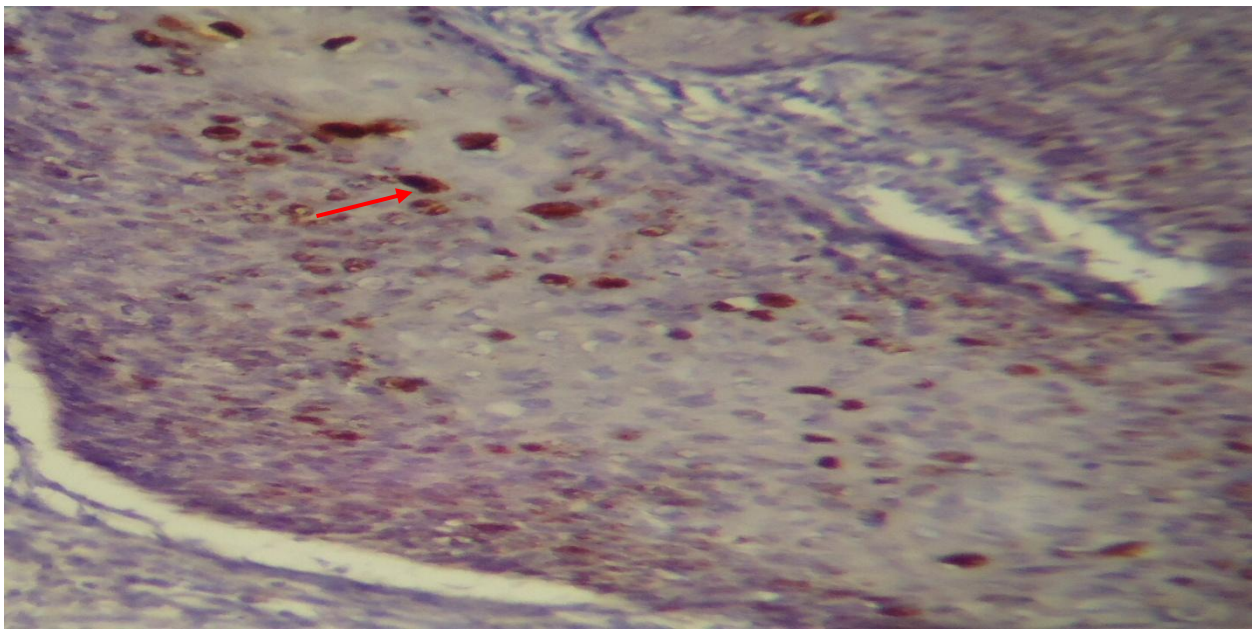

Figure 3.12: GenPoint™ HPV ISH in cervical tissues involved by Cervical carcinoma show nuclear stained of HPV (X40).

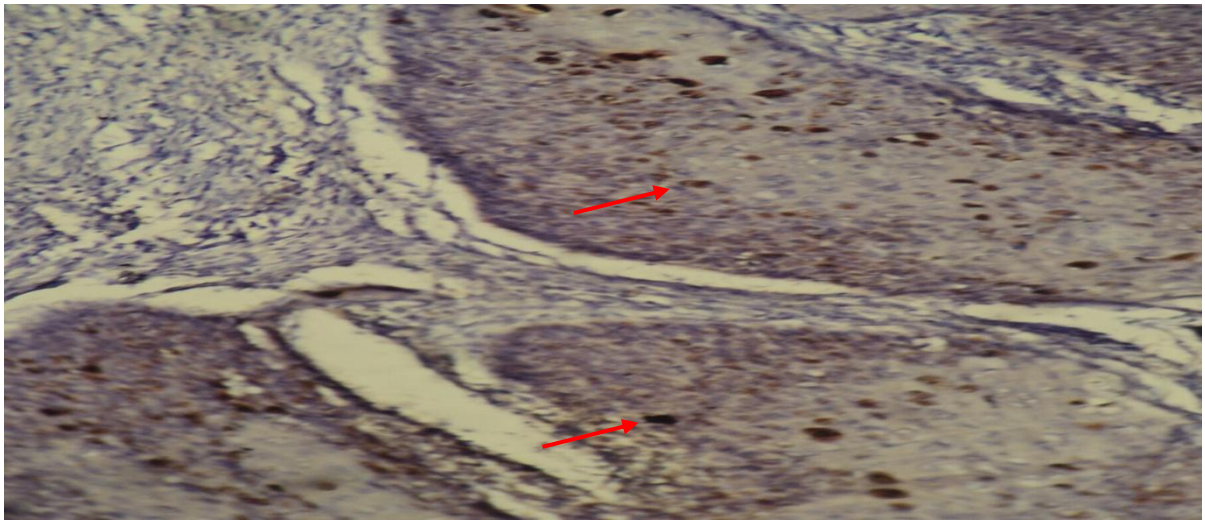

Figure 3.13: Wide Spectrum HPV ISH in cervical tissues involved by Cervical carcinoma show nuclear stained of HPV (X40).

Table 3.8: ISH Detection of HPV (GenPoint™) in benign and malignant cervical tissue.

Type of tissue		HPV-CISH (GenPoint™)		Total
		Positive	Negative	
Benign	Count	0	32	32
	%within tissue type	0%	100%	100%
Malignant	Count	17	26	43
	%within tissue type	39.5%	60.5%	100%
Total	Count	17	58	75
	% within tissue type	22.7%	77.3%	100%

(P<0.0001 , r=0.467)

Table 3.9: ISH Detection of wide spectrum HPV in benign and malignant cervical tissue.

Type of tissue		HPV- CISH (wide spe.)		Total
		Positive	Negative	
Benign	Count	0	32	32
	%within tissue type	0%	100%	100%
Malignant	Count	15	28	43
	%within tissue type	34.9%	65.1%	100%
Total	Count	15	58	75
	% within tissue type	20%	80%	100%

)P<0.0001 , r=0.431(

Table 3.10: ISH Detection of HPV (GenPoint™) in different histological types of cervical carcinoma.

Histological type		HPV-CISH (GenPoint™)		Total
		Positive	Negative	
Squamous cell carcinoma	Count	17	23	40
	%within histological type	42.5%	57.5%	93%
Adenocarcinoma	Count	0	3 100%	3

	%within histological type	0%		7%
Total	Count	17	26	43
	%within histological type	39.5%	61.5%	100%

(P<0.05 , r>0.3)

Table 3.11: ISH Detection of HPV wide spectrum in different histological types of cervical carcinoma.

Histological type		HPV- CISH (wide spe.)		Total
		Positive	Negative	
Squamous cell carcinoma	Count	15	25	40
	%within histological type	37.5%	62.5%	93%
Adenocarcinoma	Count	0	3	3
	%within histological type	0%	100%	7%
Total	Count	15	28	43
	%within histological type	34.9%	65.1%	100%

(P<0.05 , r>0.3)

Table 3.12: The relation of ISH Detection of HPV(GenPoint™) and age of the patients.

Age group		HPV-CISH (GenPoint™)		Total
		Positive	Negative	
21-30	Count	1	1	2
	%within age group	50%	50%	100%
31-40	Count	3	3	6
	%within age group	50%	50%	100%
41-50	Count	11	14	25
	%within age group	44%	56%	100%
51-60	Count	2	5	7
	%within age group	28.6%	71.4%	100%
61-70	Count	0	3	3
	%within age group	0%	100%	100%
Total	Count	17	26	43
	%within age group	39.5%	60.5%	100%

(P>0.05 , r=0.228)

Table 3.13: The relation of ISH Detection of wide spectrum HPV and age of the patients.

Age group		HPV-CISH(wide spec.)		Total
		Positive	Negative	
21-30	count	0	2	2
	%within age group	0%	100%	100%
31-40	Count	3	3	6
	%within age group	50%	50%	100%
41-50	Count	10	15	25
	%within age group	40%	60%	100%
51-60	Count	2	5	7
	%within age group	28.6%	71.4%	100%
61-70	Count	0	3	3
	%within age group	0%	100%	100%

Total	Count	15	28	43
	%within age group	34.9%	65.1%	100%

(P>0.05 , r=0.114)

Table3.14: The relation of ISH Detection of HPV (GenPoint™) and grades of tumors.

Grade of tumor		HPV-CISH(genpiont)		Total
		positive	Negative	
Grade I	Count	15	14	29
	%within tumor grade	51.7%	48.3%	100%
Grade II	Count	2	9	11
	%within tumor grade	18.2%	81.8%	100%
Grade III	Count	0	3	3
	%within tumor grade	0%	100%	100%
Total	Count	17	26	43
	%within tumor grade	39.5%	60.5%	100%

(P>0.05 , r=0.365)

Table 3.15: The relation of ISH Detection of wide spectrum HPV and grades of tumors.

Grade of tumor		HPV-CISH(wide spec.)		Total
		positive	Negative	
Grade I	Count	13	16	29
	%within tumor grade	44.8%	55.2%	100%
Grade II	Count	2	9	11
	%within tumor grade	18.2%	81.8%	100%
Grade III	Count	0	3	3
	%within tumor grade	0%	100%	100%
Total	Count	15	28	43
	%within tumor grade	34.9%	65.1%	100%

(P>0.05 , r=0.312)

Table 3.16 : The relation of ISH Detection of HPV (GenPoint™) and size(T) of tumors.

Stage(T) of tumor		HPV-CISH(genpiont)		Total
		Positive	Negative	
Ta	Count	2	4	6
	%within tumor stage(T)	33.3%	66.7%	100%
T1	Count	4	8	12
	%within tumor stage(T)	33.3%	66.7%	100%
T2	Count	3	7	10
	%within tumor stage(T)	30%	70%	100%
T3	Count	3	5	8
	%within tumor stage(T)	37.5%	62.5%	100%
T4	Count	5	2	7
	%within tumor stage(T)	71.4%	28.6%	100%

Total	Count	17	26	43
	%within tumor stage(T)	39.5%	60.5%	100%

(P>0.05 , r=0.213)

Table 3.17 :The relation of ISH Detection of wide spectrum HPV and size(T) of tumors.

Stage(T) of tumor		HPV-CISH(wide spec.)		Total
		Positive	Negative	
Ta	Count	2	4	6
	%within tumor stage(T)	33.3%	66.7%	100%
T1	Count	3	9	12
	%within tumor stage(T)	25%	75%	100%
T2	Count	3	7	10
	%within tumor stage(T)	30%	70%	100%
T3	Count	3	5	8
	%within tumor stage(T)	37.5%	62.5%	100%

T4	Count	4	3	7
	%within tumor stage(T)	57.1%	42.9%	100%
Total	Count	15	28	43
	%within tumor stage(T)	34.9%	65.1%	100%

(P>0.05 , r=0.177)

3.4. Correlation between IHC expression of HPV (anti-HPV) and CISH (genpiont) results

3.4.1. Correlation between IHC expression of HPV (anti-HPV) and CISH (genpiont) results regarding age of patients

Two cases and one case of those in age group (21-30 years) were positive for HPV in IHC result (anti-HPV) and CISH result (GenPoint™) respectively. 3 cases of those in age group (31-40 years) were positive for each of HPV in IHC result (anti-HPV) and CISH result (GenPoint™).12 cases and 11 cases of those in age group (41-50 years) were positive for HPV in IHC result (anti-HPV) and CISH result (GenPoint™) respectively. One case and two cases of those in age group (51-60 years) were positive for HPV in IHC result (anti-HPV) and CISH result (GenPoint™) respectively .None of those in age group (61-70 years) were positive for HPVs.

There was significant difference between the expression of HPV in IHC result (anti-HPV) and CISH result (GenPoint™) among these age groups (P<0.05). A

positive strong correlation between expression of these biomarkers with the age of patients was found (r=0.9) (Table 3.18).

3.4.2. Correlation between IHC expression of HPV(Anti-HPV) and CISH results (GenPoint™) regarding stage(T) of cervical carcinoma

Two cases (33.3%) out 6 cases of Ta overexpress Anti-HPV and Genpiont-HPV biomarkers, 7(58.33) and 4(33.33%) out of 12 cases of T1 showed positive HPV immunoexpression and HPV (GenPoint™) in situ hybridization respectively , 3 (30%) out of 10 cases of T2 overexpress Anti-HPV and (GenPoint™) biomarkers. 2 cases (25%) and 3 (37.50%) out of 8 cases of T3 showed positive for HPV immunoexpression and (GenPoint™) in situ hybridization respectively , and 4 (57.14%) and 5(71.43%) out of 7 cases of T4 showed positivity for HPV immunoexpression and (GenPoint™) in situ hybridization respectively. There was no significant correlation between the detection rate of HPV immunohistochemical and in situ hybridization regarding the size (T) (P>0.05), but there was very significant correlation in stage T3 and T4 of cervical carcinoma (R=0.745, r=0.730) (Table 3.19) .

3.4.3. Correlation between IHC expression of HPV (Anti-HPV) and CISH results (GenPoint™) in relation to tumor grade

Grade I cervical carcinoma cases show 14 (77.8%) and 15(88.2%) cases out of 29 cases positive for HPV immunoexpression and (GenPoint™) in situ hybridization respectively. 3 cases (16.7%) and 2 (11.8%) out of 1 cases grade II showed positive for HPV immunoexpression and (GenPoint™) in situ hybridization

respectively, while 1 (5.6%) out of 3cases grade III showed positive immunostaining for HPV and none cases of grade III showed positive for (GenPoint™) in situ hybridization. There was significant difference between the expression of HPV in IHC result (anti-HPV) and CISH result (GenPoint™) and the degree of differentiation ($P<0.05$). There was a positive correlation in well differentiated cervical carcinoma ($r=0.586$) (Table 3.20) (Figure 3.18) .

Table 3.18: Correlation between expression of IHC (anti-HPV) and CISH result (GenPoint™) regarding age of patients.

Age group	Anti-HPV immunoexpression		Genpiont-HPV Chromogenic in situ hybridization	
	Positive	Negative	Positive	Negative
21-30	2 (100%)	0 (0%)	1 (50%)	1 (50%)
31-40	3 (50%)	3 (50%)	3 (50%)	3 (50%)
41-50	12 (48%)	13 (52%)	11 (44%)	14 (56%)
51-60	1	6	2	5

	(14.3%)	(85.7%)	(28.6%)	(71.4%)
61-70	0 (0%)	3 (100%)	0 (0%)	3 (100%)
Total	18 (41.9%)	25 (58.1%)	17 (39.5%)	26 (60.5%)

(P<0.05 , r=0.9)

Table 3.19:Correlation between Anti-HPV and (GenPoint™) in relation to tumor stage(T).

Size (T) of tumor	Anti-HPV immunoexpression		HPV(GenPoint ™) Chromogenic in situ hybridization	
	Positive	Negative	Positive	Negative
Ta	2 (33.3%)	4 (66.7%)	2 (33.3%)	4 (66.7%)
T1	7 (58.3%)	5 (41.7%)	4 (25%)	8 (75%)
T2	3 (20%)	7 (70%)	3 (20%)	7 (70%)
T3	2 (25%)	6 (75%)	3 (37.5%)	5 (62.5%)
T4	4 (57.1%)	3 (42.9%)	5 (71.4%)	2 (28.6%)

Total	17 (39.5%)	26 (60.5%)	18 (41.9%)	25 (58.1%)

(P>0.05 , R>0.3)

Table 3.20:Correlation between Anti-HPV and (GenPoint™) in relation to tumor grade.

Grade of tumor	Anti-HPV immunoexpression		HPV(GenPoint™) Chromogenic in situ hybridization	
	Positive	Negative	Positive	Negative
Grade I	14 (48.3%)	15 (51,7%)	15 (51.7%)	14 (48.3%)
Grade II	3 (27.27%)	8 (72.73%)	2 (18.18%)	9 (81.82%)
Grade III	1 (33.33%)	2 (66.67%)	0 (0%)	3 (100%)
Total	18 (41.9%)	25 (58.1%)	17 (39.5%)	26 (60.5%)

(P< 0.05 , r=0.542)

3.5. Coexpression of HPV in IHC result (anti-HPV) and CISH result (GenPoint™)

3.5.1 . Coexpression of IHC expression of HPV(anti-HPV) and CISH results (GenPoint™) in relation to tumor grade

None of grade III cervical carcinoma cases expressed HPV in IHC result (anti-HPV). Positive expression for both biomarkers revealed 11 cases of grade I and 2 cases of grade II, positive IHC result (anti-HPV) alone revealed 3 cases of grade I, one case of grade II and one cases of grade III and positive CISH result (GenPoint™)alone revealed four cases of grade I and one case of grade II. However, cases of neither expression revealed 11 cases of grade I, 7 cases of grade II and 2 cases of grade III.

There was a significant difference among these groups of IHC result and CISH result regarding tumor grade ($P<0.05$)with a strong correlation toward well differentiated grades ($r=0.586$) .

Regarding tumor grades, there was a significant correlation between simultaneous and separate, and neither expression of HPV in IHC result (anti-HPV) and CISH result (GenPoint™) ($P<0.05$) with a positive generally correlation with tumor grade could be noticed ($r>0.3$)(Table 3.21).

3.5.2. Coexpression of IHC expression of HPV(anti-HPV) and CISH results (GenPoint™) in relation to the size (T) of tumor

Positive expression of both biomarkers revealed two cases of Ta, 3 cases of T1, 2 cases of T2, 2 cases of T3 and 4 cases of T4. Positive immunoexpression of HPV alone revealed 1 cases of Ta, 4 cases of T1, 1 case of T2, none of T3, none of T4. Positive CISH for HPV alone revealed none of Ta, 1 case of T1, 1 case of T2, 1 case

of T3, 1 case of T4, while cases of neither expression revealed three cases of Ta, 4 cases of T1, 6 cases of T2, 5 of T3, 2 cases of T4. There was no significant difference among the expression of these biomarkers regarding size (T) of tumor (P>0.05). However, a positive correlation with increasing age was detected in those coexpressing both biomarkers (r>0.6).

Regarding tumor sizes, there was a significant correlation between simultaneous and separate, and neither expression of HPV in IHC result (anti-HPV) and CISH result (GenPoint™) (P<0.05) with a positive generally correlation with tumor size (T) could be noticed (r >0.3)(Table 3.22).

3.5.3. Coexpression of IHC expression of HPV(anti-HPV) and CISH results (GenPoint™) in relation to age of patients

Cervical carcinoma cases positive for both biomarkers revealed that one case was seen in each of age group (21-30 years) and (51- 60 years) . Two cases were seen in age group (31-40 years), nine cases were seen in age group (41-50) and none in age group (61-70 years).

Cases positive for IHC result(anti-HPV) alone revealed that one case was seen in each of age group (21-30 years) and (31-40), 3 cases in age group (41-50 years), none cases in each of age group (51-60 years) and (61-70 years). Cases positive for CISH result (genpiont) alone revealed that none was seen in age group (21-30 years) , one case was seen in age group (31-40 years), 2 cases in age group (41-50 years), and one case in age group (61-70 years), while cases of neither expression

revealed that none cases in age group (21-30 years), 2 cases in age group (31-40 years), 11 cases in age group (41-50 years), 5 cases in age group (51-60 years), and 4 cases in age group (61-70 years). There was a significant difference among the expression of these biomarkers regarding age of the patients ($P < 0.05$). However, a significant correlation with increasing age was detected in those coexpressing both biomarkers ($r>0.3$) (Table 3.23).

Table 3.21:Coexpression of IHC result(anti-HPV) and CISH result (GenPoint™) in relation to tumor grade.

Grade of tumor	Both (anti-HPV and GenPoint™) positive	Only (anti-HPV) positive	Only GenPoint™ positive	Both (anti-HPV and GenPoint™) negative	Total	P value	R test
Grade I	11 (84.6%)	3 (60%)	4 (80%)	11 (55%)	29 (67.4%)	P<0.05	r=0.58
Grade II	2 (15.4%)	1 (20%)	0 (20%)	8 (35%)	11 (25.6%)	P>0.05	r=0.54
Grade III	0 (0%)	1 (20%)	0 (0%)	2 (10%)	3 (7%)		
Total	13 (30.3%)	5 (11.6%)	4 (9.3%)	21 (48.8%)	43 (100%)		

Table 3.22 :Coexpression of IHC result(anti-HPV) and CISH result (Gen- Point™) in relation to the size (T) of tumor

Stage of tumor	Both (anti-HPV and Gen-Point™) positive	Only (anti-HPV) positive	Only Gen-Point™ positive	Both (anti-HPV and Gen-Point™) negative	Total	P value	R test
Ta	2 (15.4%)	0 (0%)	0 (0%)	4 (19%)	6 (13.9%)	P>0.05	r=0.667
T1	3 (23%)	4 (80%)	1 (25%)	4 (19%)	12 (27.9%)	P>0.05	r=0.354
T2	2 (15.4%)	1 (20%)	1 (25%)	6 (28.7%)	10 (23.3%)	P>0.05	r=0.542
T3	2 (15.4%)	0 (0%)	1 (25%)	5 (23.8%)	8 (18.6%)	P>0.05	r=0.745
T4	4 (30.8%)	0 (0%)	1 (25%)	2 (9.5 %)	7 (16.3%)	P>0.05	r=0.730
Total	13 (30.3%)	5 (11.6%)	4 (9.3%)	21 (48.8%)	43 (100%)		

Table 3.23 : Coexpression of IHC result(anti-HPV) and CISH result (genpiont) in relation to age of patients.

Age group	Both (anti-HPV and GenPoint™) positive	Only (anti-HPV) positive	Only GenPoint™ positive	Both (anti-HPV and GenPoint™) negative	Total
21-30	1 (7.7%)	1 (20%)	0 (0%)	0 (0%)	2 (4.7%)
31-40	2 (15.4%)	1 (20%)	1 (25%)	2 (9.5%)	6 (14%)
41-50	9 (69.2%)	3 (60%)	2 (50%)	11 (52.4%)	25 (58%)
51-60	1 (7.7%)	0 (0%)	1 (25%)	5 (23.8%)	7 (16.3%)
61-70	0 (0%)	0 (0%)	0 (0%)	3 (14.3%)	3 (7%)
Total	13 (30.3%)	5 (11.6%)	4 (9.3%)	21 (48.8%)	43 (100%)

(P> 0.05, r > 0.3)

Table 3.24 : The intensity of HPV immunohistochemical expression of cervical cancer in correlation to grade & size of tumor.

Parameters	Total number Of patients	The intensity of HPV-immunostaining				
		Score 0	Score 1+	Score2+	Score3+	Score 4+
Tumor grade						
I	29	15	2	3	4	5
II	11	8	1	1	0	1
III	3	2	0	0	0	1
Tumor size						
Ta	6	4	1	1	0	0
T1	12	5	1	2	2	2
T2	10	7	0	0	1	2
T3	8	6	0	0	1	1
T4	7	3	1	1	0	2

(P>0.05 , r=0.342)

Table 3.25 : The intensity of HPV Genpiont ™ expression of cervical cancer in correlation to grade & size of tumor

Parameters	Total number Of patients	The intensity of HPVgenpiont- CISH staining				
		Score 0	Score 1+	Score 2+	Score 3+	Score 4+
Tumor grade						
I	29	14	3	3	4	5
II	11	9	0	1	0	1
III	3	3	0	0	0	0
Tumor size						
Ta	6	4	1	1	0	0
T1	12	8	1	1	1	1

T2	10	7	0	0	1	2
T3	8	5	0	1	1	1
T4	7	2	1	1	1	2

(P>0.05 , r=0.342)

Parameters	Total number Of patients	The intensity of HPV wide spectrum - CISH staining				
		Score 0	Score 1+	Score 2+	Score 3+	Score 4+
Tumor grade						
I	29	16	2	4	3	4
II	11	9	0	0	1	1
III	3	3	0	0	0	0
Tumor size						
Ta	6	4	1	0	1	0

T1	12	9	1	1	0	1
T2	10	7	0	1	1	1
T3	8	5	0	1	1	1
T4	7	3	0	1	1	2

Table 3.26 : The intensity of HPV wide spectrum expression of cervical cancer in correlation to grade & size of tumor.

(P>0.05 , r=0.342)

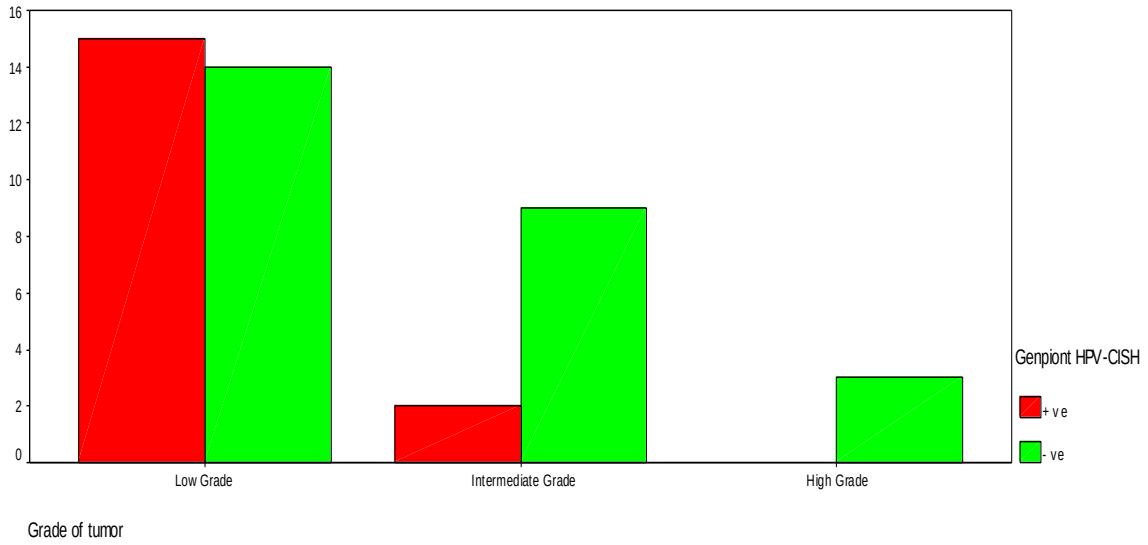

Figure 3.14: The relation of ISH Detection of HPV Genpiont ™ and tumor grade.

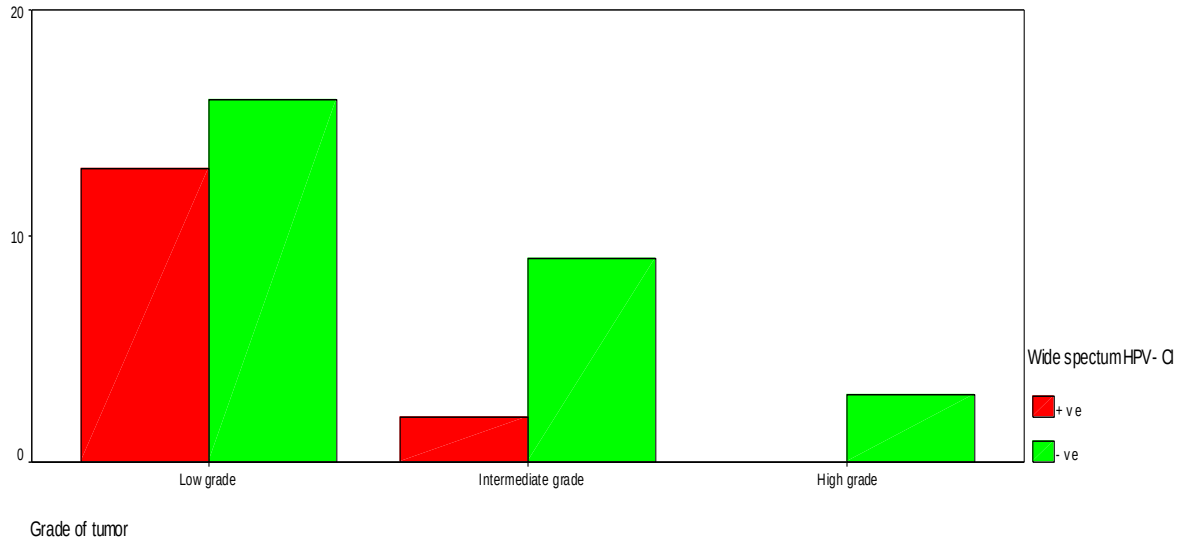

Figure 3.15: The relation of ISH Detection of wide spectrum HPV and tumor grade .

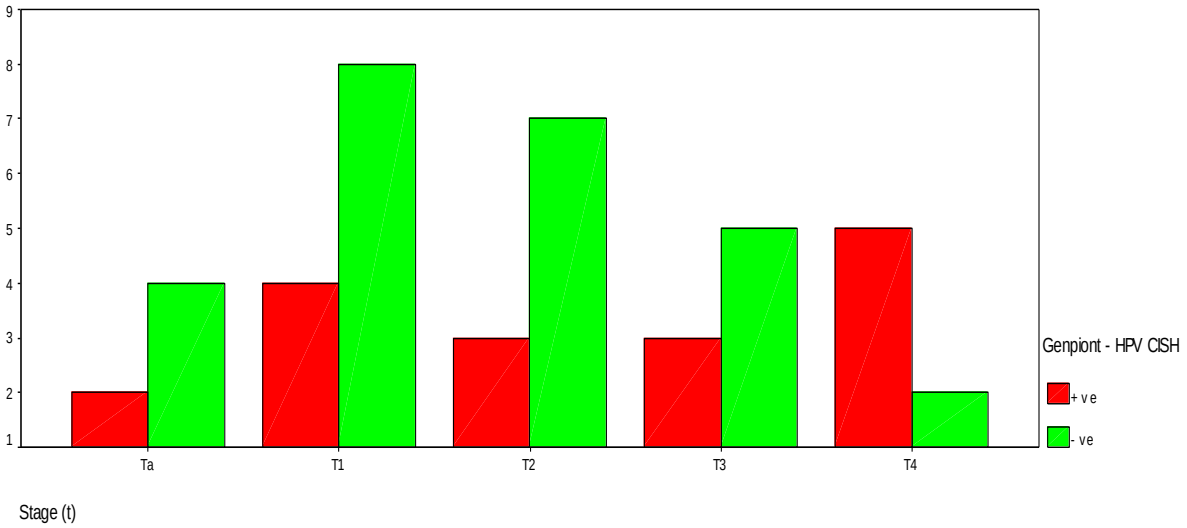

Figure 3.16: The relation of ISH Detection of HPV Genpiont ™ and size(T) of tumors.

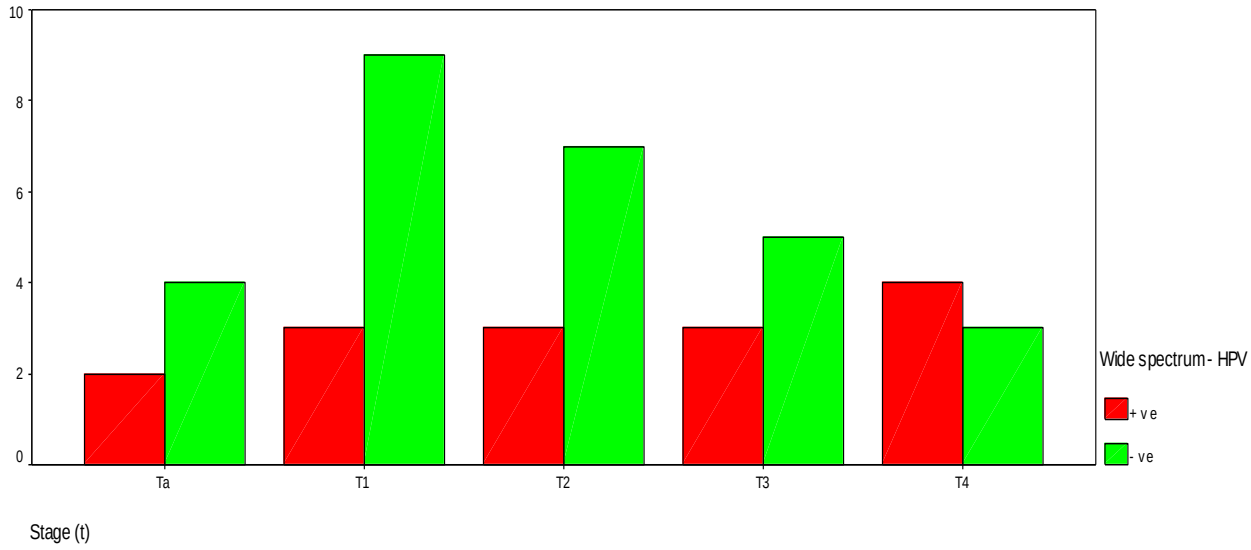

Figure 3.17: The relation of ISH Detection of wide spectrum HPV and size(T) of tumors.

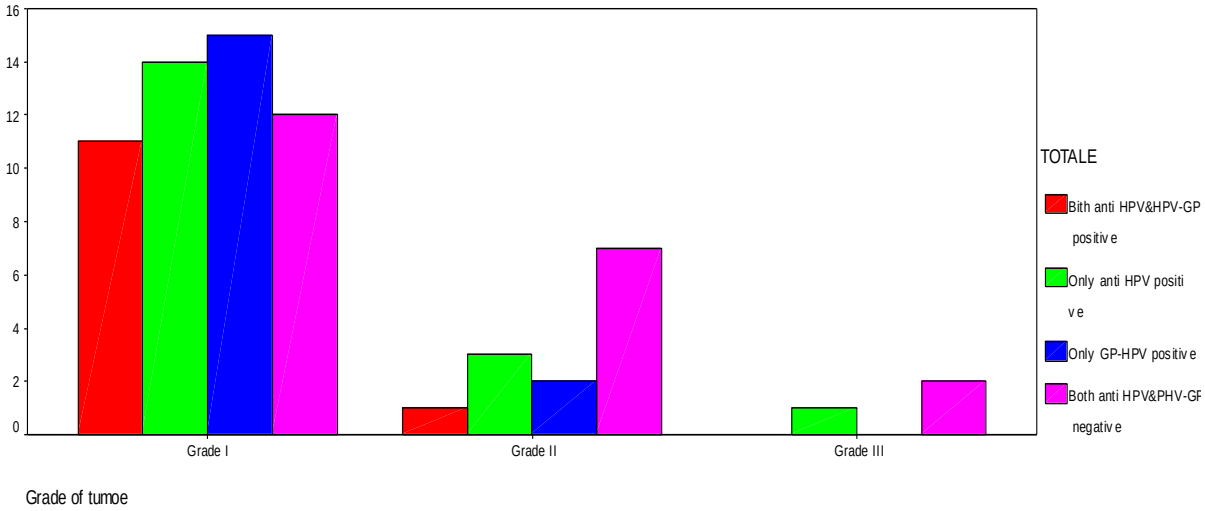

Figure 3.18: Correlation between Anti-HPV and HPV Genpiont ™ in relation to tumor grade.

4. Discussion

The present study represents the fundamental and leading step conducted in our region for detecting and understanding the role of HPV in cervical carcinoma. Up to our knowledge , there was no published paper studied the efficiency of both immunohistological and CISH technique in detection of HPV in different clinicopathological parameters of cervical cancer patients in our region (middle of Iraq).

We designed this study using an advanced generation of in situ hybridization as molecular technique and immunohistochemistry technique to compare the efficiency of both techniques. They are effective methods to detect and localize HPV within the affected tissues.

4.1. Study population

A total of 75 cervical tissue samples were included in this study. 32 cases comprised a normal control group and the remaining 43 cases comprised a cervical carcinoma (Table: 3.1). Cervical tissues obtained for diagnosis are routinely stored in pathology archives. These samples are an increasingly important resource for the study of disease. The tissues are well-characterized by classical histopathology, and blocks can be selected to be representative of the disease. Also, the retrospective analysis permits rapid correlation of assay results

with clinical outcome [187] and for diseases with low prevalence, such as invasive cervical cancer. The accrual of cases is often inadequate for prospective analysis.

The age of patients which ranged between 28-65 years with a Mean=46.02±7.960 (Mean ± SD) years. 2 patients (4.65%) were within the 2nd decade of life and more , while 6 patients (13.95%) were within the 3th decade , 25 cases (58.14%) within the 4th decade,7 patients (16.28%) within 5th decade and only 3 cases (6.98%) were within the 6th decade. Most HPV infections occur in young adults with a peak at 35-45 years of age. Currently, HPV infections have reached epidemic proportions in young, sexually active population. The mean age of patients within the mean age of carcinoma in situ and invasive cervical cancer was 30 and 50 years respectively[43, 191].

4.2. HPV immunohistochemical study

4.2.1. HPV in the presented benign and malignant cervical tissue

Regarding the assessment of the control group which includes normal cervical tissue (control group), all cases were negative for HPV immunostaining without any expression of even faint cytoplasmic stain with significant difference from malignant cases.

Conversely, in the current study the results have clarified that 18 out of 43 cases of cervical carcinoma were expressing HPV immunohistochemical nuclear staining in their histological sections. However, malignant cases showed 41.9% positivity

for HPV within this type of tissue and 100% within immunostaining of HPV with a significant difference comparing with normal and benign cases (P<0.05).

HPV immunoexpression is exclusively reported in malignant cervical tissue, this finding agrees with study reported by **Al-Shwaikh *et al*.,(2006)** [189].

Furthermore, our results are higher than **AL-Azawi *et al.*** [190] results (41.9% Vs 33.3%) with no significant difference. Nevertheless, it reflects trait of increasing rate of HPV infection in our population, this could partially be attributed to the major changes in the Iraqi society that began to occur in the last years might lead to increased opportunities for exposure to human papillomavirus (HPV) infection. In another part ,other causative agents with oncogenic potentials could have a role in the carcinogenesis of HPV-negative cervical cancers.

4.2.2. HPV immunoexpression and grade of tumor

The immunohistochemical analysis of the results revealed that HPV immunoexpression was noticed in 48.3% within grade I cervical carcinoma, 27.3% within grade II cervical carcinoma. In grade III, 33.3% of them revealed positive within grade of tumor. There was no significant difference among these grades (P value = 0.463) (Table 3.6).

A high proportion of **HPV** overexpression was reported in well and moderately differentiated cervical cancer, while less proportion of **HPV** was reported in poorly differentiated cervical cancer. These results are in agreement with result of previous similar study such as **(AL-Azawi *et al.* 2006)** [190].

The carcinoma of uterine cervix had been associated with HPV types only distantly related to the known genital HPVs and undetectable under the experimental condition. It is possible that HPV particles can get lost if other genetic changes in cancer cells take over the role of HPV [26,110].

4.2.3. HPV immunoexpression and size (T) of tumor

The immunohistochemical analysis of the results revealed that HPV immunoexpression in the presented cervical carcinoma was noticed in 33.3% of Ta, 58.3% of T1 , 30% of T2 , 25% of T3 and in 57.1% of T4. In our study, all sizes of the presented cervical carcinoma were no significantly correlated to the immunostaining of HPV (R =0.006), without any significant difference in the immunostaining of HPV among them (P value=0.467) (Table 3.7). It is possible that HPV functions are no longer necessary. Thus, it may be those specific HPVs play a part in early intraepithelial stages of tumor progression and that viral DNA sequence can be lost at later stages **[26,110]**.

4.2.4. HPV immunoexpression and age

The results of **HPV** immunoexpression in relation to age distribution of patients revealed that **HPV immunoexpression** was detected in (100%) in age group 21-30 years, (50%) in 31-40 years, 48% in 41-50 and 14.3% 51-60 years without any

significant difference among these age groups (P>0.05). However, it looks positively well correlated to the age of patient (R = 0.392).

The possible contribution of a cohort effect due to the major changes in our society that began to occur in the last years may lead to increased opportunities for exposure to human papillomavirus (HPV) infection at an early age.

Age is an important determinat of risk of HPV infection. The greatest risk of HPV infection coincides with greatest metaplastic activity which occurs at puberty and first pregnancy, and declines after menopause [43,44] .

4.2.5. HPV and histological types of cervical carcinoma

Two different types of cervical carcinoma reported in this study were adenocarcinoma and squamous cell carcinoma.

HPV immunoexpression of these two different types was as follows; none of adenocarcinoma was reported to be HPV positive, while 45% of squamous subgroups of cervical carcinoma were HPV positive with a significant difference among the two variants (p< 0.05).

HPV GenPoint™ in situ hybridization of these two different types was as follows; none of adenocarcinoma was reported to be HPV positive, while 42.5% of squamous subgroups of cervical carcinoma were HPV positive with a significant difference among the two variants (p< 0.05).

HPV wide spectrum in situ hybridization of these two different types was as follows; none of adenocarcinoma was reported to be HPV positive, while 37.5%

of squamous subgroups of cervical carcinoma were HPV positive with a significant difference among the two variants ($p< 0.05$).

Most researches studied the expression of HPV in cervical carcinoma have focused on the squamous carcinoma subgroup. This may reflect that most cervical cancers arise at the squamo-columnar junction where continuous metaplastic changes occur which increased the opportunities for exposure to human papillomavirus (HPV) infection [43,44].

4.3. In situ hybridization (ISH) technique

4.3.1. ISH Detection and Genotyping of HPV in benign and malignant Cervical tissue

Forty-three archival tissue blocks with cervical carcinoma were subjected to ISH technique to detect HPV and genotype.

Regarding the assessment of the control group which includes normal cervical tissue, all cases were negative for HPV in situ hybridization detection without any expression of even faint cytoplasmic stain with significant difference from malignant cases.

Conversely, in the current study the results have clarified that 17 out of 43 cases of cervical carcinoma were expressing HPV GenPoint™ in situ hybridization nuclear staining in their histological sections. However, malignant cases showed 39.5% positivity for HPV GenPoint™ within type of tissue with a significant

difference comparing with normal and benign cases (P<0.05). Also the results have clarified that 15 out of 43 cases of cervical carcinoma were expressing wide spectrum HPV in situ hybridization nuclear staining in their histological sections. However, malignant cases showed 34.9% positivity for wide spectrum HPV within type of tissue with a significant difference comparing with normal and benign cases (P<0.05).

The prevalence of HPV DNA in the total group was 39.5% (17 out of 43) and 34.9% (15 out 43) within chromogenic in situ hybridization of HPV (GenPoint™) and wide spectrum HPV respectively .

These results point out that HPV immunoexpression is exclusively reported in malignant cervical tissue. It is similar to study reported by **Al-Shwaikh *et al.*,(2006)[(189)].** So, HPV is a landmark for malignant cervical tissues and does not play role in normal cervical tissues .

4.3.2. ISH Detection of HPV DNA and grade of tumor

The results of chromogenic in situ hybridization (CISH) technique revealed that the prevalence of **HPV(high risk)** was noticed in 51.7% within grade I of cervical carcinoma, 18.2% within grade II of cervical carcinoma, while none cases of grade III of cervical carcinoma revealed positive for HPV. There was no significant difference among these grades (P >0.05). However, it is significantly correlated to the grade of tumor (r = 0.365) (Table 3.13).

The prevalence of wide spectrum HPV was noticed in 44.8% within grade I of cervical carcinoma, 18.2% within grade II of cervical carcinoma, while none cases

of grade III of cervical carcinoma revealed positive for HPV. There was no significant difference among these grades (P value<0.05). However, it is significantly correlated to the grade of tumor (R = 0.312).

In the present study, the prevalence of HPV is more frequently associated with well and moderately differentiated than poorly differentiated. This result is similar to the result point out by **AL-Azawi *et al.,* (2006)** [(190)] **.**

Moreover, in human, the carcinoma of uterine cervix had been associated with HPV types. In appropriation of HPV DNA-negative cervical cancers in this study, it may be that specific HPVs play a part in early intraepithelial stages of tumor progression and that viral DNA sequence can be lost at later stages. This was confirmed by absence of HPV in lymph node metastases of HPV-positive primary invasive cervical cancer. Thus, it is possible that HPV-DNA can get lost if other genetic changes in cancer cells take over the role of HPV [(26,110)].

4.3.2. ISH Detection of HPV DNA and size(T) of tumor

The analysis of HPV DNA Detection revealed that the expression of HPV(high risk) was noticed in 33.3% of Ta, 33.3% of T1, 30% of T2, 37.5% of T3 and in 71.4% of T4 (Table 3.16).

The amplification of wide spectrum HPV was noticed in 33.3% of Ta, 25% of T1, 30% of T2, 37.5% of T3 and in 57.1% of T4. From these readings of HPV amplification. It looks that as the size of tumor increases, more HPV CISH detection will be noticed(Table 3.17).

However, in our study, all the sizes of the presented cervical carcinoma were no significant difference in the CISH of HPV among them (P>0.05). It is possible that HPV functions are no longer necessary. Thus, it may be those specific HPVs play a part in early intraepithelial stages of tumor progression and that viral DNA sequence can be lost at later stages or it is possible that HPV-DNA can get lost if other genetic changes in cancer cells take over the role of HPV [(26,110)].

4.3.4. ISH Detection of HPV DNA and age of patients

HPV GenPoint™ amplification in relation to age distribution of patients revealed that HPV DNA was detected in 50% of age groups 21-30, 50% of age group 31-40 years ,44% of age group 41-50 years , 28.6% of age group 51-60 years without significant difference among these age groups (P>0.05). While Wide Spectrum HPV amplification in relation to age distribution of patients revealed that HPV DNA was detected in 50% of age group 31-40 years ,40% of age group 41-50 years, 28.6% of age group 51-60 years without significant difference among these age groups (P>0.05).

4.3.5. Prevalence of HPV DNA in FFPE - cervical tissue

The HPV DNA was detected by ISH with non istotopic labelled probe. The available probes for HPV detection consisted of a mixture of HPV probes:

GenPoint™ HPV Biotinylated DNA probe has been found to react with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 on formalin-fixed paraffin embedded cervical tissues and/or cells by in situ hybridization and The Wide Spectrum HPV Biotinylated DNA Probe hybridizes to anogenital human papillomaviruses (HPV) including types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, and 52. ISH for HPV DNA was carried out on all 75 specimens. All normal cases showed no specific signals for HPV DNA, 17 (39.5 %) of 43 cases of carcinoma of cervix uteri and they were positive for GenPoint™ HPV DNA. 15 (34.9%) of 43 cases carcinoma of cervix uteri showed to be positive for Wide Spectrum HPV DNA.

This study has found that immunodetection of HPV in 41.9% of 43 of cervical carcinoma cases, while the CISH detection of HPV in cervical carcinoma cases ranges from 34.9% to 39.5%. It reflects evidence of increasing rate of HPV infection in our population. This may be reflect the variant genetic make-up in different ethnic groups or may be an effect of environmental damaging agents.

Furthermore, the findings of the presented study showed relatively higher prevalence of HPV compared to the previous work carried out in our country (33.3%). Though there was no significant difference was found between the two studies, the possible contribution was due to either the differences in the sample sizes, the period of sample collection or the sensitivity of the Kits used.

The detection of different oncogenic HPV types in all grades of cancer could be related to HPV genetic variability that could account for the wide-spectrum of pathology found in the associated lesions. Moreover, the association of particular HPV variants with specific histological types of cervical carcinoma suggests or

different cellular factors that influence viral ability to transform cell. Many studies performed in Non-Moslem western populations, a higher prevalence of HPV than the prevalence reported in our study. The prevalence of HPV DNA in cervical lesions ranges from 25 to 90 %. HPV-16 accounts for the highest proportion, followed by HPV-18, HPV-45 and HPV-31, but their incidence varies depending on the country [188] .

The results of ISH for HPV DNA detection in squamous cell carcinoma 17(42.5%) of 40 cases of squamous cell carcinoma of cervix uteri showed to be positive for GenPoint™ HPV DNA ,while 15(37.5%) of 40 cases of squamous cell carcinoma of cervix uteri showed to be positive for Wide Spectrum HPV DNA. The results of the present study show that high oncogenic risk HPV genotype constitutes the majority of the HPV genotypes identified. Therefore, these HPV genotypes could play an important causal role in these cases of cervical squamous cell carcinoma.

These results are in agreement with result of previous similar studies such as **(Al-Shwaikh *et al.*,(2006)[189] in Iraq).** The results of the present study show none one of cases of adenocarcinoma positive for GenPoint™ HPV DNA or Spectrum HPV DNA. They are not in agreement with the result obtained by **AL-Azawi *et at*.(2006)[190].** This may be reflect that most cervical cancers arise at the squamo-columnar junction where continuous metaplastic changes occur which increased opportunities for exposure to human papillomavirus (HPV) infection[43,44].

4.4. Association between ISH and immunohistochemical Detection of HPV(high risk) in cervical carcinoma

In this study, we investigate the prevalence of HPV in cervical carcinoma by two methods of detection: the chromogenic in situ hybridization (CISH) and immunohistochemical techniques.

Chi-square test was used to assess associations between immunodetection of HPVs and ISH detection of the virus . A significant correlation was found between immunohistochemical detection of HPVs and CISH detection of HPVs in the cases of cervical carcinoma examined using Spearman's correlation test ($p < 0.05$, $R=0.6$). Immunostaining and CISH for HPVs were not detected in any of the cases of normal epithelium , while in malignant lesions HPVs overexpression was accumulated in cell nuclei.

The results of immunohistochemical technique revealed that the prevalence of HPV protein was noticed in 41.9% of cervical carcinoma within histological types of tissue. The results of chromogenic in situ hybridization (CISH) technique revealed that the prevalence of HPV DNA was noticed in 39.5% of cervical carcinoma within histological types of tissue.

Chi-square test showed that there was a highly significant association between HPV overexpression by both methods to histological type of tissue ($p < 0.001$, $r=0.567$).

Immunohistochemical analysis of HPV protein overexpression and CISH analysis of HPV DNA overexpression in relation to the age group of cervical carcinoma patients revealed that the frequency of positive HPV was slightly increasing with increased age up to 50 years old, but it starts to decrease in older age group. It seems that the detection rate is increasing as the age increases. Chi-

square test showed that there was a highly significant association between the detection rate of immunohistochemistry and CISH to the age of patient ($p<0.05$, $r = 0.306$).

4.5. Correlation between IHC expression of HPV and CISH results

In our study, a positive immunostaining of HPV was reported in 77.8% in grade I cervical cell carcinoma, while CISH result of HPV was positive in 88.2% of the same grade. In grade II, IHC result of HPV was positive in 16.7% of cases compared with 11.8% positive CISH result of HPV in the same grade. Finally, grade III showed 5.6% positive IHC result of HPV while none of cases of the same grade showed a positive CISH result of HPV. Most of positive IHC result of HPV in cervical cancer were positive for HPV in CISH result. There was a strong positive correlation between IHC result and CISH result regarding the grades ($r = 0.586$) but with significant difference among these grades (P value =0.003) (Table 3.20) (Figure 3.7).

Regarding the stage (T) of cervical cell carcinoma, positive staining of HPV in IHC and CISH techniques were reported to be equally expressed in Ta (33.3% for each). In T1-cervical cell carcinoma, HPV positive stain was reported in 58.3% IHC against 33.3% CISH, HPV was reported to be equally expressed in T2 (30% for each), T3 HPV was 25% IHC against 37.5% CISH, and in T4 HPV was 57.14 IHC% against 71.43% CISH. There was a strong positive correlation between the detection rate of HPV immunohistochemical (IHC) and in situ hybridization (ISH) regarding the stage of tumor ($r > 0.3$). There was a significant correlation in stage

T3 and T4 of cervical carcinoma (r=0.745,r=0.730) but without any significant difference among these different sizes (P value = 0.313) (Table 3.19) .

Regarding the age of patients, a positive staining of HPV in IHC and CISH techniques was reported in 50% and 100% of age group 21-30 years. HPV positive stain was reported to be equally expressed in age group 31-40 (5o% for each). In age group 41-50, HPV was reported in 48% IHC against 44% CISH . While in age group 51-60, HPV was 14.3% IHC against 28.6% CISH. None of those in age group (61-70 years) were positive for HPVs. There was a positive correlation between the detection rate of HPV immunohistochemical(IHC) and in situ hybridization (ISH) regarding age of patients (r = 0.3). There was significant difference among these age group (P < 0.05).

In general, there was a strong correlation between the detection rate of HPV in IHC results and CISH results in cervical carcinoma regarding the grade, stage, and age. Up to our knowledge, there is no published paper worldwide that studied the detection rate of HPV correlations between IHC and CISH techniques in cervical cell carcinoma.

4.6. Coexpression of IHC result (anti-HPV) and CISH result (genpiont) and grade of tumor

We found that **IHC (anti-HPV)** and **CISH (genpiont)** were coexpressed in 84.6% of cases of grade I cervical carcinoma , in 15.4% of cases of grade II coexpress both biomarkers, and in none of grade III cervical carcinoma have any coexpress.

The co-expression of **IHC (anti-HPV)** and **CISH (genpiont)** was significantly correlated with grade of tumor (R=0.586, P>0.05)(Table 3.21).

A 7.7% of cases of age group 21-30 years, 15.4% of 31-40, 69.2% of 41-50 and 7.7% of 51-60 were coexpressing both biomarkers. The coexpression cases showed a positive correlation with the age group (R=0.745) without a significant difference between these groups (P<0.05) (Table 3.23).

A 15.4% of cases of Ta cervical carcinoma, 23% of T1, 15.4% of T2, 15.4% of T3 and 30.8% of T4 were coexpressing both biomarkers. The coexpression cases showed a positive correlation with tumor size (R=0.745) without a significant difference between these groups (P<0.05) (Table 3.22).

The coexpression of both biomarkers of IHC(anti-HPV) and CISH (genpiont) seems to be strongly positively correlated with age; as the age of patient increases, the coexopression increases ,with a significant difference (R>0.3)(P<0.05) .

Conclusions

From the above results, the following can be concluded:

1. Normal cervical tissues did not show any expression of HPV DNA or protein in both techniques (IHC and CISH) .

2. The prevalence of HPV in cervical carcinoma in our region is lower than other studies in western countries , and it is increasing in our population in the last few years.

3. The expression rate of HPV DNA and proteins were detected in 39.5% and 41.9% of cervical cell carcinoma ,respectively.

4. The using of chromogenic in situ hybridization and immunohistochemistry techniques are well correlated methods for detection of HPV in cervical carcinoma .

5. The expression of HPV in cervical carcinoma have focused on the squamous carcinoma subgroup. While none of adenocarcinoma was reported to be HPV positive.

Recommendations

On the bases of the above results and conclusions, the following can be recommended:

1. Further concurrent genetic DNA analysis of HPV gene in cervical cancer that could detect HPV genotypes not detectable by the present ISH technique to clarify the precise prevalence of oncogenic HPV types in cervical cancer.

2. Larger study including all types of cervical cancer for further investigation HPV overexpression and determining the relation between HPV genotypes and progression of cervical carcinoma.

3. Further studies with a large number of cases are needed to study the actual prevalence of HPV infection among Iraqi general population and attendants of dermatological and sexually transmitted diseases clinics for better implications of HPV role in many important lesions and cancer.

4. Since a national Iraqi screening program for early detection and treatment of cervical neoplasia/dysplasia is designed and recently employed, it is crucial to complement this program by adding in situ hybridization HPV DNA probe or PCR testing for a precise assessment and better cost effectiveness.

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