IMMUNOCYTOCHEMICAL AND MOLECULAR ANALYSIS OF LIQUID-BASED CERVICAL CYTOLOGY SPECIMENS IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NORTHERN NIGERIA

BY

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FACULTY OF BASIC CLINICAL SCIENCES

COLLEGE OF HEALTH SCIENCES

AHMADU BELLO UNIVERSITY

ZARIA

AUGUST, 2018

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DEPARTMENT OF PATHOLOGY

FACULTY OF BASIC CLINICAL SCIENCES

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AUGUST, 2018

DECLARATION

I declare that the thesis entitled: 'IMMUNOCYTOCHEMICAL AND MOLECULAR ANALYSIS OF LIQUID-BASED CERVICAL CYTOLOGY IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NORTHERN NIGERIA' was carried out by me in the Department of Pathology, Faculty of Basic Clinical Sciences, College of Health Sciences of Ahmadu Bello University Zaria under the supervision of Prof. A. Mohammed, Prof. A.H. Rafindadi and Prof. M.A. Abdul. The information derived from the literature were duly acknowledged in the text and a list of references provided. No part of this thesis was presented for another degree or diploma at any university.

_____ _____

SaadAliyu Ahmed Date

CERTIFICATION

This thesis entitled: 'IMMUNOCYTOCHEMMICAL AND MOLECULAR ANALYSIS OF LIQUID-BASED CERVICAL CYTOLOGY SPECIMENS IN AHMADU BELLO UNIVERSITY TEACHING HOSPITAL ZARIA, NORTHERN NIGERIA' by SaadAliyu AHMED was carried out in the Department of Pathology, Faculty of Basic Clinical Sciences, College of Health Sciences of the Ahmadu Bello University Zaria under our supervision.

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ABSTRACT

Cervical cancer is a common and important public health problem for adult women in developing countries. Many industrialized countries have achieved significant successes in reducing invasive cervical cancer burden over the past six decades mainly due to introduction of screening programmes. On the other hand, cervical cancer is not only the second most common malignancy in adult women, but is also the most common malignancy of the female genital tract in sub-Saharan Africa.The aim of this study was to analyse liquid-based cervical cytology specimens among women attending Family Planning/Gynaecology Clinics in Ahmadu Bello University Teaching Hospital (ABUTH), Zaria using immunocytochemical and molecular techniques.Five hundred (500) consenting women between the ages of 18 – 59 years attending the Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital Zaria, Nigeria were recruited consecutively. Cervical smears were reported using the Bethesda System and all abnormal smears had HPV DNA testing done after extraction of the DNA. The extracted HPV DNA was amplified using Polymerase Chain Reaction (PCR) with GP5+/GP6+ and MY09/MY11 consensus primers which amplifies a 150bp fragment of the L1 Hr-HPV genomic region and positive ones had genotyping done by sequencing. Dual immunocytochemical staining using p16INK4a and Ki67 were done on LSIL and borderline cases. All cases classified as high grade had biopsy and histology done.Five hundred (500) women were screened and the mean age was 40.5 ± 9.7 years. The prevalence of abnormal smears was 5% and the cytological diagnoses comprise Negative (95.0%), Low Grade Squamous Intraepithelial Lesion (LSIL, 2.6%), High Grade Squamous Intraepithelial Lesion (HSIL, 1.4%) and Atypical Squamous Cells of Undetermined Significance (ASC-US, 1.0%). Hr-HPV DNA was prevalent among subjects with abnormal smears andtypes 16, 18 and 33were the most prevalent. No case of co-infection was seen in this study. The evaluation of p16INK4a and Ki67 biomarkers in LSIL

and borderline smears revealed that 33.3% of cases were positive and subsequently reclassified to HSIL. All the associated risk factors examined were all found to be within the 95% confidence interval but only parity and STDs were found to be statistically significant ($P<0.05$).Abnormal smears are not uncommon and cytology was found to have good sensitivity and specificity to detect them. One third of LSIL/borderline cases were reclassified high grade due to positivity to p16INK4a/ki67 dual stain. Mass screening for cervical cancer should be encouraged by the State and Federal governments to come up with reliable prevalence from the community and dual immunocytochemical stain with p16/Ki67 should be considered in triage for LSIL and borderline lesions of the cervix. HPV vaccination against the most common subtypes are encouraged among the target population.

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ABBREVIATIONS

ABUTH	Ahmadu Bello University Teaching Hospital
ACCP	American College of Clinical Pharmacy
AGUS	Atypical Glandular Cells of Undetermined Significance
ASCUS	Atypical Squamous Cells of Undetermined Significance
CIN	Cervical Intraepithelial Neoplasia
DAB	3,3-Diaminobezidine
ELISA	Enzyme-Linked Immunosorbent Assay
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HR-HPV	High Risk Human Papillomavirus
HSIL	High Grade Squamous Intraepithelial Lesion
IUD	Intrauterine Device
LBC	Liquid-Based Cytology
LSIL	Low Grade Squamous Intraepithelial Lesion
NILM	Negative for Intraepithelial Lesion or Malignancy
PCR	Polymerase Chain Reaction
TBS	The Bethesda System
VIA	Visual Inspection with Acetic Acid

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Cervical cancer is a common and important public health problem for adult women in developing countries (Sahasrabuddhe, 2011; Omran and AlSheeha, 2015). Many industrialized countries have achieved significant successes in reducing invasive cervical cancer burden over the past six decades, and with annual incidence rates between 4 and 14 per 100,000 (Sahasrabuddhe, 2011). Cervical cancer no longer ranks among the top ten cancers in these settings. The low incidence is achieved through substantial healthcare investments for screening programmes and diagnostic workup in these countries. On the other hand, cervical cancer is the leading cancer among women in many resource-constrained settings of the developing world, where incidence and mortality rates are about five- to six-times higher (Bouvard*et al.,* 2009). Rates are highest in sub-Saharan Africa, SouthEast Asia and parts of South America, where CervicalCancer represents from a sixth up to a fifth of all cancers among women (Sahasrabuddhe, 2011). Cervical cancer is not only the second most common malignancy in adult women, but is also the most common malignancy of the female genital tract in Nigeria (Mohammed, 2006; Ahmed, 2013).

Known risk factors for cancer of the cervix include persistent infection with high risk Human Papillomavirus (HPV), early age at first intercourse and multiple sexual partners. A male consort who in turn has had intercourse with multiple women also confers a significant risk (Arevian, 1997;Omran and AlSheeha, 2015). Other risk factors of cancer of the cervix include cigarette smoking and immune suppression, especially those who have undergone renal transplantation; this may be mediated by the Human Papilloma Virus (HPV). HIV infection may increase women's risk for cervical neoplasia. It is claimed that the vast majority

of cervical cancers could be prevented if all women were offered and complied with high quality cytological screening programmes. (Bakheit, 2004)

HPV is the most common sexually transmitted infection in the United States, affecting both men and women (Arney and Bennett, 2010). Currently, there are many different known HPV genotypes that have been grouped into low-risk and high-risk categories and designated as causing mucosal or cutaneous infections (de Villers, 2004). Warts are generally the result of infection by low-risk types of HPV, including 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108. High-risk types of HPV include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Ahmed*et al*., 2015). The high-risk strains induce cervical dysplasia and can lead to the development of several types of cancers including cancer of the cervix, vulva, vagina, anus, and penis. The most common of these is HPV-associated cervical cancer (Walboomers, 1999; Ahmed *et al*., 2015). HPV types 16 and 18 are the most prevalent worldwide (Manga*et al*., 2015).

Papanicolaou (Pap) staining is the gold standard for detecting abnormal cervical epithelial cells, using microscopic analysis of conventional cervical smears or cell suspensions from liquid cytology medium. Currently, cervical examinations and Pap tests remain the screening method of choice for most women in many parts of Africa. Morphological findings from a cytology analysis determine the level of risk for developing cervical malignancy. Molecular diagnostic tests for HPV can augment screening for cervical cancer when used in conjunction with the Pap smear (Molijn, 2005).

The cervical cytology is reported using The Bethesda System (TBS) of classification which established the 2-tiered reporting system forsquamous intraepithelial lesions (SILs): Low-grade Squamous Intraepithelial Lesion, LSIL (CIN I) and High-grade Squamous Intraepithelial Lesion, HSIL (CIN II and CIN III). This terminology reflectedthe up-to-date

understanding of human papillomavirus (HPV) biology – squamous epithelium is affectedby the virus in essentially 2 ways: either as viral infectionor as viral-associated precancer (NCI, 1989; Nayar and Wilbur, 2015). Due to the inherent limitations of morphologic interpretation;borderline cases, the Atypical Squamous Cells of Undetermined Significance' ASCUS (ASC-US and ASC-H)and Atypical Glandular Cells of Undetermined Significance (AGUS) were introduced and previously posed significant clinical managementproblems, leading to significantcolposcopic follow-up and/or treatment of these women (Nayar and Wilbur, 2015; Solomon, 2015).

Colposcopy is usually done on women with abnormal smears and/or positive HR-HPV test.However, a newer concept of triage using biomarkers p16INK4a and Ki67 dual immunostaining has been advocated to avoid overreferral for colposcopy. P16INK4a is an efficient triage method; the dual staining with Ki67 was introduced mainly to increase reproducibility and specificity compared with stand-alone P16INK4a staining (Allia, 2015). Diffuse p16INK4a immunostaining is the hallmark of HSIL (CIN2 and CIN3) regardless of HPV status (Zhang, 2015).

Normally, the p16 protein triggers cellcycle arrest in the course of cellular differentiation processes and israrely observed simultaneously with Ki-67. However, in transformingHPV infections, p16 is strongly overexpressed in proliferatingcells, and the p16-induced cell cycle arrest is circumvented byinactivation of pRb, which is mediated by the expression of theviral E7 oncoprotein of high-risk HPV types. Observing dualexpression therefore suggests HPV-induced deregulation of the cellcycle and may be used as an indicator for the presence of high grade lesions (Uijterwaal*et al*., 2014).

1.2 Statement of the Research Problem

In developed countries, an estimated 15,000 new cases of cervical cancer and 5,000 deaths occur annually from the disease (Bakemeier, 1995).These relatively low figures are a far cry from those obtainable in Nigeria where an estimated 250/100,000 cases and 155,000 deaths are recorded annually (Pisani, 1999; Shehu and Sule, 2005). Globally, women particularly those living in developing countries suffer higher rates of morbidity and mortality from cervical cancer than previously noted (Pisani, 1999).

There are several socio-cultural practices like early marriage and polygamy, particularly in Northern Nigeria which have been suggested to promote the development of cervical cancer (Avidime*et al*., 2014). However, infection with high risk HPV is a necessary cause of cervical cancer worldwide (Walboomers, *et al*., 1999). Among the many known HPV types, about40 can infect the cervix out of which 13 types (16, 18,31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66) from amongthe alpha species have been classified as group 1 carcinogens(Schiffman,*et al*., 2007; McLaughlin-Drubin, *et al*., 2008). Of these oncogenic viruses, HPV-16 andHPV-18 are the most prevalent worldwide. They havebeen associated with about 70 % of invasive cervical cancerworldwide and about 50 % of HSIL(McLaughlin-Drubin,*et al.*, 2008). Aetiologically,five HPV types (16, 18, 31, 33, and 45) have been linkedto 80 % of cervical cancers while the other types contributethe remaining 20 % (Veldhuijzen,*et al.*, 2010). Infection of the genitaltract with HPV is considered the most common sexuallytransmitted infection worldwide. Persistent HPV infection of the cervical epithelium isthe major risk factor for cervical cancer as more than99 % of cases contain hr-HPV (Manga*et al*., 2015).

HPV-driven molecular mechanisms underlying the development of cervical lesions have provided a number ofpotential biomarkers (like p16INK4a and Ki67) for both diagnostic and prognostic use in the clinical management of these women (Hwang and Shroyer, 2012).

The prevalence of pre-invasive lesions in Nigeria is relatively high and considering the fact that the most common abnormal diagnosis being ASCUS, the need for triage becomes imperative in order to reduce over-referral for colposcopy, treatment and cost. Despite the high level of morbidity and mortality in Nigeria there is general lack ofcervical cancer screening policy for the general public. Where screening takes place it is usually opportunistic and lacks coordinated approach in the management of abnormal smears (Ahmed, *et al*., 2013; Avidime, *et al*., 2014).

1.3 Justification for the Study

The value of screening for cervical cancer has been proven (Sankaranarayanan*et al*., 1999). Such screening in developed countries has achieved a decrease in incidence and mortality by about 80% (Avidime, 2014). Although screening facilities are available in many parts of developing countries, the incidence of cervical cancer remains very high and many patients present with late stage disease (Ferley, 2001).It has been suggested that detecting the HPV DNA is more sensitive test forcervical cancer early detectionthan Pap cytology. It is used in addition toconventional Pap cytology in certain settings and currently introducedas an alternative for primary screening in many industrializedcountries (Schiffman*et al*., 2011). However, considering the highprevalence of HPV infections particularly in youngwomen, detecting HPV DNA is a poorly specific test forreal cellular alterations and therefore requires additionaltriage tests for the specific identification of women needingfurther work-up or treatment (Saslow*et al*., 2012). In order to reduce referral rates, unnecessarytreatments and finally costs, new biomarkers (p16INK4a and Ki67) have been identified and suggested to improve diagnosis of cervical cancer andits precancerous lesions (McLaughlin-Drubin*et al*., 2011; Reuschenbach*et al*., 2013).

Cervical cancer screening that involve Cytology, HPVDNA testing and genotyping; and cellular biomarkers will be the first of its kind in women attending the Family planning/Gynaecology clinics of the Ahmadu Bello University Zaria. This study will also deploy research interest in this area.

1.4 Aim and Objectives

1.4.1 Aim: To analyse liquid based cervical cytology specimens among women attending Family planning/Gynaecology clinics in Ahmadu Bello University Teaching Hospital(ABUTH) Zaria using immunocytochemical and molecular techniques.

1.4.2 Objectives

1. To determine the prevalence of pre-invasive lesions on cervical smears among Family Planning and Gynaecology Clinic attendees in Ahmadu Bello University Teaching Hospital, Zaria using the Bethesda Classification.
2. To evaluate p16INK4a and Ki67 biomarkers using immunocytochemistry in LSIL and borderline lesions in cervical smears among women attending Family Planning and Gynaecology clinics of Ahmadu Bello University Teaching Hospital Zaria.
3. To compare high grade cervical smear lesions with histological diagnosis among women attending Family Planning and Gynaecology clinics of Ahmadu Bello University Teaching Hospital, Zaria.
4. To determine the prevalence of HighRisk Human Papillomavirus (Hr-HPV)DNA in pre-invasive lesions on cervical smears among women attending Family Planning and Gynaecology Clinics ofAhmaduBello University Teaching Hospital, Zaria.

5. To determine the molecular subtypes of Hr-HPV in pre-invasive lesions on cervical smearsamong women attending Family Planning and Gynaecology Clinics of AhmaduBello University Teaching Hospital, Zaria.
6. To determine the association between pre-invasive lesions on cervical smears and possible associated risk factors for cervical canceramong women attending Family Planning and Gynaecology clinics of AhmaduBello University Teaching Hospital, Zaria.

1.5 Research Questions

The following were the research questions raised:

1. What is the prevalence of pre-invasive lesions of the cervix among Family Planning and Gynaecology Clinic attendees in the Ahmadu Bello University Teaching Hospital, Zaria?
2. How important is p16INK4a/Ki67 dual stain in evaluating LSIL and borderline lesions of the cervix among women attending Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital, Zaria?
3. Is there any correlation between cytology and histology in high grade lesions among women attending Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital Zaria?
4. What is the prevalence of Hr-HPV in pre-invasive lesions of the cervix among women attending Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital, Zaria?
5. What are the molecular subtypes of Hr-HPV in pre-invasive lesions of the cervix in women attending Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital, Zaria?

6. What are the associated risk factors for cervical cancer among women attending Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital, Zaria?

CHAPTER TWO

2.0 LITERATURE REVIEW

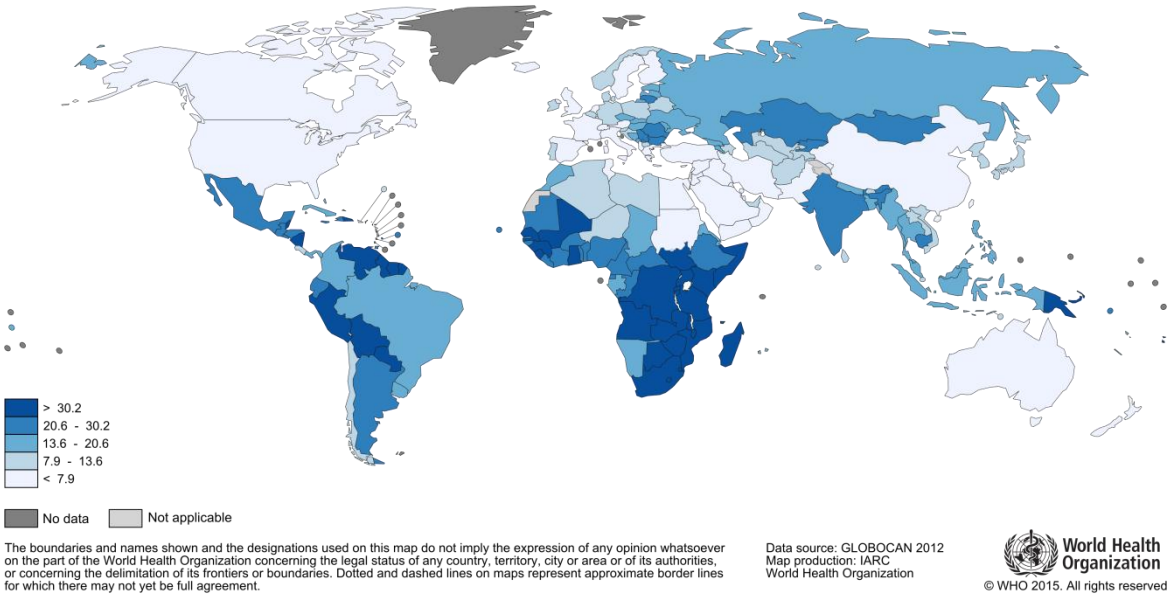
2.1 Epidemiology of Cervical Cancer

Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases in 2012 (GLOBOCAN 2012). As with liver cancer, a large majority (around 85%) of the global burden occurs in the less developed regions, where it accounts for almost 12% of all female cancers. High-risk regions, with estimated ASRs over 30 per 100,000, include Eastern Africa (42.7), Melanesia (33.3), Southern (31.5) and Middle (30.6) Africa. Rates are lowest in Australia/New Zealand (5.5) and Western Asia (4.4) as seen in Figure 2.1. Cervical cancer remains the most common cancer in women in Eastern and Middle Africa (GLOBOCAN, 2012).

There were an estimated 266,000 deaths from cervical cancer worldwide in 2012, accounting for 7.5% of all female cancer deaths. Almost nine out of ten (87%) cervical cancer deaths occur in the less developed regions. Mortality varies 18-fold between the different regions of the world, with rates ranging from less than 2 per 100,000 in Western Asia, Western Europe and Australia/New Zealand to more than 20 per 100,000 in Melanesia (20.6), Middle (22.2) and Eastern (27.6) Africa as seen in Figure 2.2 (GLOBOCAN, 2012).

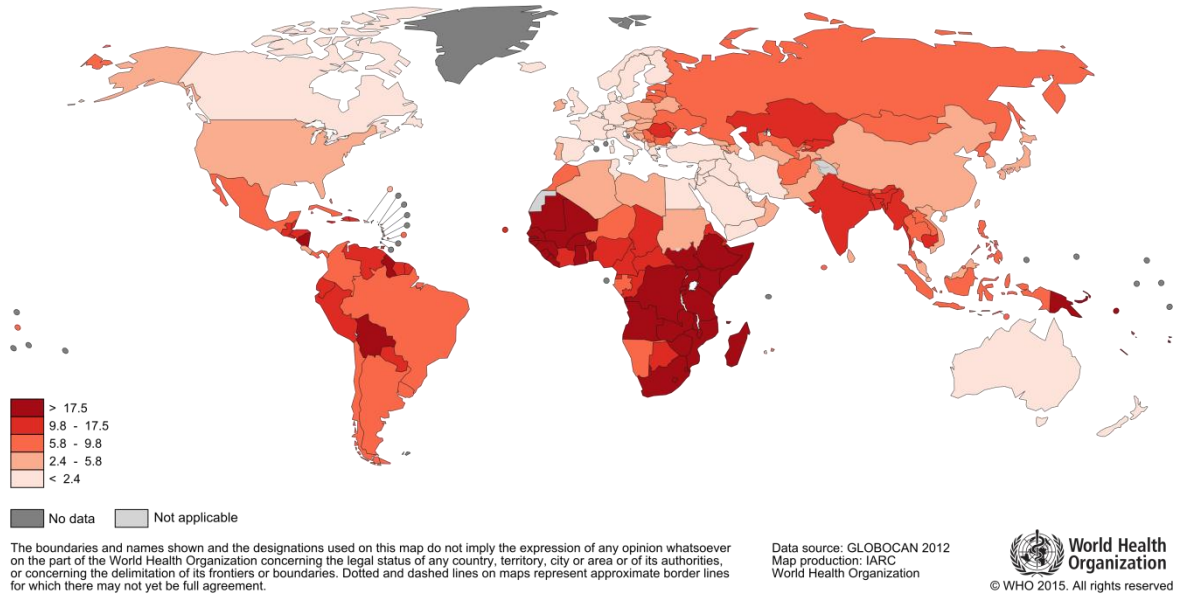
Approximately 85% of new cases of cervical cancer occur in developing countries and 80%-90% cervical cancer cases in developing countries occur among women age 35 and older(Sherris*et al.*, 2005). Cervical cancer progresses slowly from precancerous lesion to advanced cancer. Globally the incidence of the cancer is very low in women under age of 25 years. However, the incidence increases at age of 35 to 40 years and reaches the maximum in women in their 50s and 60s (ACCP, 2005).

In Nigeria, cancer of the cervix used to be the commonest reproductive tract cancer (Parkin, *et al*., 2003). The incidence rates show little change (ASR 20.9 in 1960-69 and 19.9 in 1998-99) and a recent study of gynaecological malignancies over a period of two decades (1976-95) based on registry data revealed a steady increase in the frequency of new cases (Babarinsa, *et al*., 1998). This reflects the poor development of screening facilities and the lack of a national screening programme.



Estimated age-standardised rates (World) per 100,000

Figure 2.1: Estimated Cervical Cancer Incidence Worldwide in 2012



Estimated age-standardised rates (World) per 100,000

Figure 2.2:Estimated Cervical Cancer Mortality Worldwide in 2012

2.2 Risk Factors of Cervical Cancer

2.2.1 Human Papillomavirus

The major risk factor for cervical cancer is infection with human papillomavirus. Fifteen HPV types were classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82); three were classified as probable high-risk types (26, 53, and 66); and 12 were classified as low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108). There was good agreement between the epidemiological classification and the classification based on phylogenetic grouping (Munoz, *et al*., 2003).

The infection with HPV genotypes has geographical variation. In the United States, HPV-16 was the most common type, but HPV-58 was the second most prevalent in Mexican population (Giuliano, *et al*., 1999). In Spain 75% of screened women were sero-positive for HPV-58 (Touze, *et al*., 2001). HPV-58 was the second most common genotype in Japan (Sasagawa, *et al*., 2001). In China the prevalence of HPV-58 was 24% (Chan, *et al*., 1999). In Paraguay, HPV- 58 was detected in 2.7% of cervical carcinomas (Rolon, *et al*., 2000). In Brazil prevalence rates of HPV-16, HPV- 58, HPV-31 and HPV-18 were 49%, 13, 12% and 4.5% respectively (Camara, *et al*., 2003). The association between cervical cancer and high risk oncogenic types of HPV is clearly demonstrated (Bosch, *et al*., 1995). Women with cervical intraepithelial neoplasia III and invasive cervical cancer have high prevalence of HPV- 16. HPV-18 (Rabelo-Santos, *et al*., 2003). Huwanga, *et al*., (2004) reported that HPV-18 was present more frequently in (84.6%) adenocarcinomas and adenosquamous carcinomas. In Mali, HPV DNA was identified in 97% of cervical cancer cases, and HPV types 16, 18, and 31 were detected in 60% of cases and 45% of controls (Bayo, *et al*., 2002).

2.2.2 Smoking

Women who smoke are about twice as likely as non-smokers to get cervical cancer. Smoking exposes the body to many cancer-causing chemicals that affect organs other than the lungs. Tobacco by-products have been found in the cervical mucus of women who smoke. It is believed that these substances damage the DNA of cervix cells and may contribute to the development of cervical cancer. Smoking also makes the immune system less effective in fighting HPV infections.Long duration smoking (20 or more years) was associated with a two-fold increase in the risk of squamous cell carcinoma, but smoking was not associated with the risk of adenocarcinoma (Green, *et al*., 2003).

2.2.3 Immunosuppression

Human immunodeficiency virus (HIV), the virus that causes Acquired Immune Deficiency Syndrome (AIDS), damages the body's immune system and places women at higher risk for HPV infections. This may explain the increased risk of cervical cancer for women with AIDS(Saharabudde et al., 2011; University of Michigan, 2016).

2.2.4 Chlamydia infection

Chlamydia is a relatively common kind of bacteria that can infect the reproductive system. It is spread by sexual contact. Chlamydia infection can cause pelvic inflammation, leading to infertility. It has been reported that there has been 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)(Graham, 2010; University of Michigan, 2016).

2.2.5 Diet

Women whose diets don’t include enough fruits and vegetables may be at increased risk for cervical cancer. Overweight women are more likely to develop adenocarcinoma of the cervix(University of Michigan, 2016).

2.2.6 Oral contraceptive (birth control pills)

There is evidence that taking oral contraceptives for a long time increases the risk of cervical cancer. Research suggests the risk goes up the longer a woman takes oral contraceptives, but goes back down after stopping the pill. The American Cancer Society suggests that women discuss with their doctor whether the benefits of using oral contraceptives outweigh the potential risks.Long-term use of oral contraceptives could be a co-factor that increases risk of cervical carcinoma by up to four-fold in women who are positive for cervical HPV (Moreno, *et al*., 2002; Gierisch*et al*., 2013).

2.2.7 Intrauterine device use

A recent study found that women who had ever used an intrauterine device (IUD) had a lower risk of cervical cancer. The effect on risk was seen even in women who had an IUD for less than a year, and the protective effect remained after the IUDs were removed. Intrauterine device use is associated with cervical intraepithelial neoplasia in women that already had persistent HPV infection (Brinton, *et al.*, 1989).

2.2.8 Multiple full-term pregnancies

Women who have had 3 or more full-term pregnancies have an increased risk of developing cervical cancer. Cervical cancer risk is significantly associated with multi-parity. It was 5.1 fold for women with 14 pregnancies or more (Brinton, *et al*., 1987). In Mali, risk factor of cervical cancer for parity of 10 was 4.8 fold compared to parity of <5 children (Bayo, *et al*., 2002; Brinton, *et al.*, 1989).

2.2.9 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 2 times more likely to get cervical cancer later in life than women who waited to get

pregnant until they were 25 years or older. Younger age of having the first child is significantly associated with the risk of cervical cancer (Brinton, *et al*., 1989).

2.2.10 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 they may not get screened or treated for cervical pre-cancers.Cervical cancer incidence rates have been observed to vary between different socio-economic groups, and the importance of these factors may vary between different geographical regions (Parikh, *et al*., 2003).

2.2.11 Diethylstilbestrol (DES)

Diethylstilbestrol (DES) is a hormonal drug that was prescribed between 1940 and 1971 for some women thought to be at increased risk for miscarriages. 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. There is about 1 case of this type of cancer in every 1,000 women whose mothers took DES during pregnancy. This means that about 99.9% of "DES daughters" do not develop these cancers (Graham, 2010; University of Michigan, 2016).

2.2.12 Family history of cervical cancer

Cervical cancer may run in some families. If a mother or sister had cervical cancer, the chances of developing the disease are 2 to 3 times higher than if no one in the family had it.There is also genetic risk for cervical cancer; evidence revealed that familial clustering of cervical cancer and its precursor forms (Hemminki, *et al*., 1999; Magnusson, *et al*., 1999). Genetic susceptibility to cervical cancer is related to HLA class II. HLA, B7 and DQB1 are positively associated with cervical neoplasia while DRB1 is negatively associated with disease (Engelmark, *et al*., 2004).

2.3 Virology of Human Papillomavirus

Humanpapillomaviruses are non-enveloped double-stranded DNA viruses that infect the epithelial basal layer. The majority of HPV infections occur without symptoms and is cleared by the host within 8-12 months. However, in a small number of cases infection may persist resulting in intraepithelial neoplasia and, over time, progression to invasive carcinoma.

2.3.1 HPV taxonomy

To date, 189 papillomavirus (PV) types have been isolated. The majority have been isolated from humans but many have been found in other mammals, birds andreptiles(Bernard *et al*., 2010). The classification of the different types is based on the nucleotide sequence ofpart of the HPV genome (L1) and is divided into genera, species, and types. The amount of nucleotide sequence identity shared within this taxonomy is:genera – less than 60%, species –between60%and70%,types–between 71% and 89% as shown in Figure 2.4(de Villiers *et al*., 2004). The two main genera are the Alphaand Beta papillomaviruses, accounting forapproximately90% of the known HPV types (Doorbar, 2006). The larger Alpha genus contains the genital/mucosal HPV types. These types can be further classified into low and high risk depending on the frequency with which they are found in cancer. Table 2.1 shows the list of diseases caused by human papillomavirus infection.

Table 2.1: List of diseases caused by human papillomavirus infection(Graham, 2010)

Site	Disease
Skin	Deep plantar warts
	Common warts
	Butchers' warts
	Periungal warts
	Carcinoma and keratosis in epidermodysplasia verruciformis
Anogenital tract	Anogenital warts
	Cervical, vulvar, perianal intraepithelial neoplasia
	Carcinoma of the cervix, vulva, anus, penis
Respiratory tract	Recurrent respiratory papillomatosis
	Squamous cell carcinoma of the larynx, tonsils, tongue base, sinuses, lung
Other	Conjunctival papillomatosis
	Oesophageal cancer
	Various carcinomas associated with immunodeficiencies and HIV-related.
	Breast cancers?

Beta papillomaviruses are associated with cutaneous lesions in humans. These infections remain unnoticed amongst the general population. However, in immunocompromised individuals and patients with the inherited disease epidermodysplasia verruciformis, the virus can spread and may result in the development of non-melanoma skin cancer (Harwood and Proby, 2002, Pfister, 2003).The other HPV genera include Gamma, Mu and Nu. They are associated mainly with benign cutaneous lesions (de Villiers *et al*., 2004).

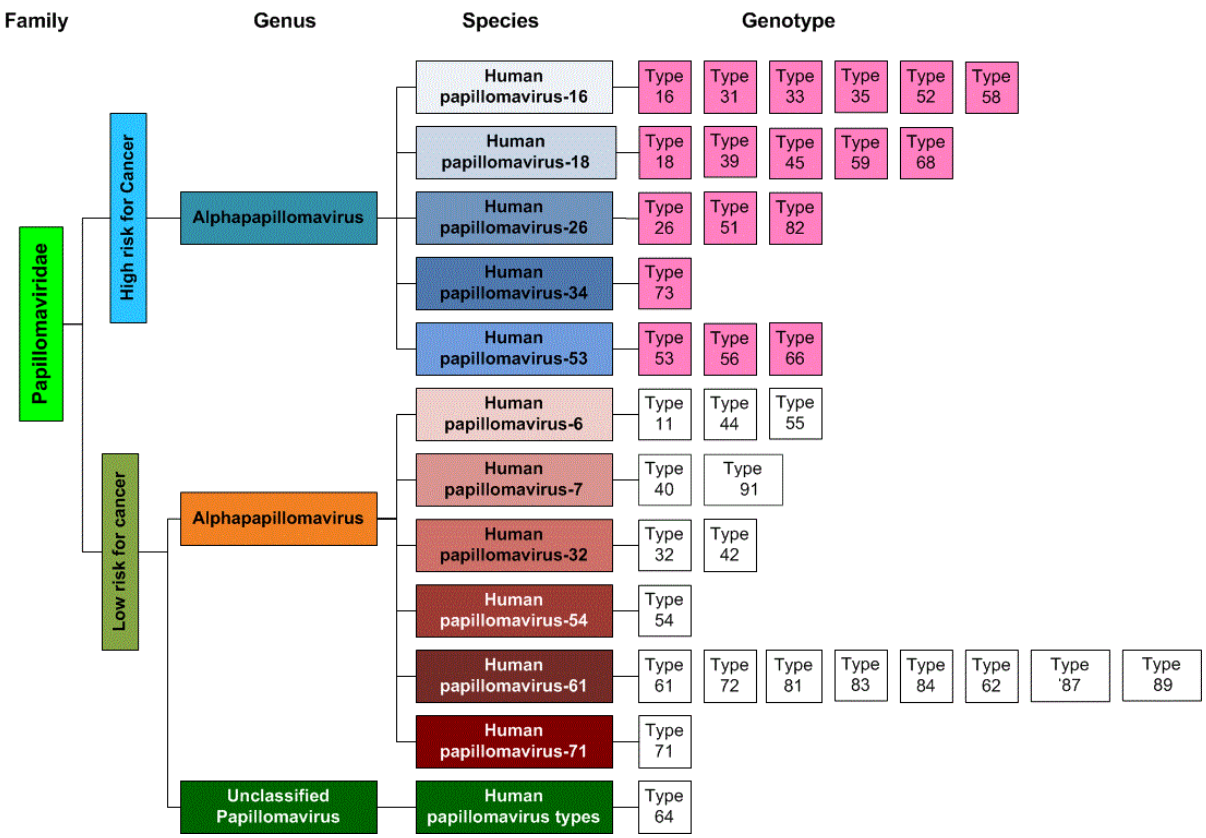


Figure 2.3: Classification of Alpha Genus of Human Papillomavirus Viruses
(Harvard, 2017)

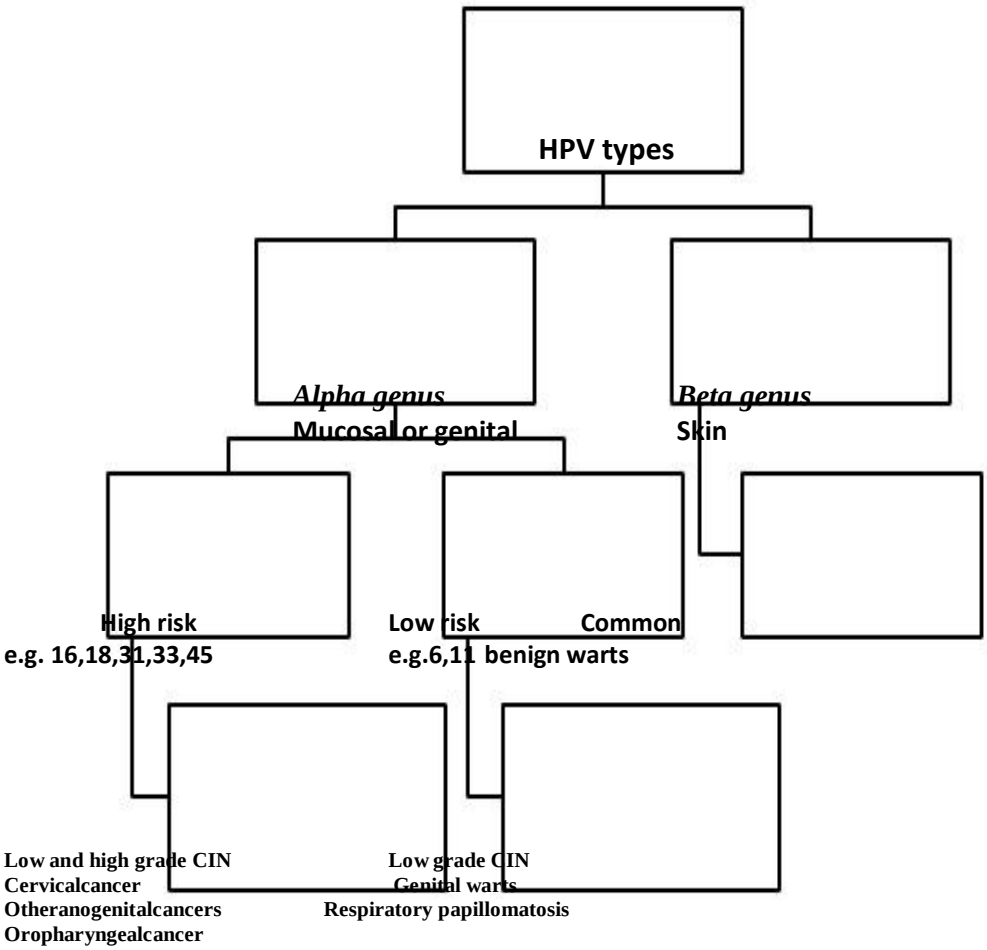


Figure 2.4: Features of the two largest papillomavirus genera (Bernard *et al.*, 2010).

2.4 Molecular Pathogenesis of Cervical Cancer

2.4.1 The HPV genome and proteins

All HPV are non-enveloped double stranded DNA viruses. Their genomes are circular andapproximately 8 kilobase pairs in size. Most encode eight major proteins, 6 located in the early" region 2 in the "late" region (Table 2.1). The "early" proteins are regulatory in function. For example, they play roles in HPV genome replication and transcription, cell cycle, cell signalling and apoptosis control, immune modulation and structural modification of the infected cellas in Figure 2.5(Chung and Gillison, 2009; Graham, 2010).

Most of these proteins are expressed throughout the infectious cycle perhaps with reduced expression at late times. E4 is the first virus protein expressed late in infection followed by the late proteins, L1 and L2, which are specifically expressed in the granular layer of the epithelium. For the mucosal epithelium, this is the outermost epithelial layer. L1 and L2 comprise the virus capsid required for virus transmission, spread and survival in the environment (Graham, 2006). To date functional studies of virus proteins have focussed on these eight proteins primarily in the HR HPV (Table 2.2). However, there is evidence for other virus proteins that may have important functions. For example, HPV16 and 18 express more than one isoform of the E6 cell regulatory oncoprotein. Four mRNA isoforms (FLE6, E6*I, E6*II, E6*X) have been observed in HPV16 infected cervical epithelial cells and two in HPV18 infection (Graham, 2006). A role for the E6*I isoform in antagonising FLE6 function has been suggested, as has opposing roles of FLE6 and E6*I in regulation of procaspase 8 in the extrinsic apoptotic pathway (Graham, *et al*., 2010).

More recently, a stand-alone function of the E6*I isoform has been determined in cellular protein degradation. Several isoforms of the virus replication/transcription factor E2 have

also been noted for a number of HPV. E2 has an N-terminal domain that mediates protein-protein interactions, a flexible hinge region and a C-terminal DNA binding domain. Truncated E2 proteins may be translated from alternatively spliced E2 RNAs to generate E1^E2 and E8^E2 protein isoforms present in HPV16 and 31-infected cells (Ammermann*et al*., 2008). These E2 isoforms may act in a dominant-negative manner to modulate the function of full length E2 (Ammermann*et al*., 2008). For example, a full length E2/E8^E2 dimer may bind DNA but fail to recruit E1 to initiate virus replication. Similarly, such a dimer may be unable to interact with cellular transcription factors to alter virus genome transcription.

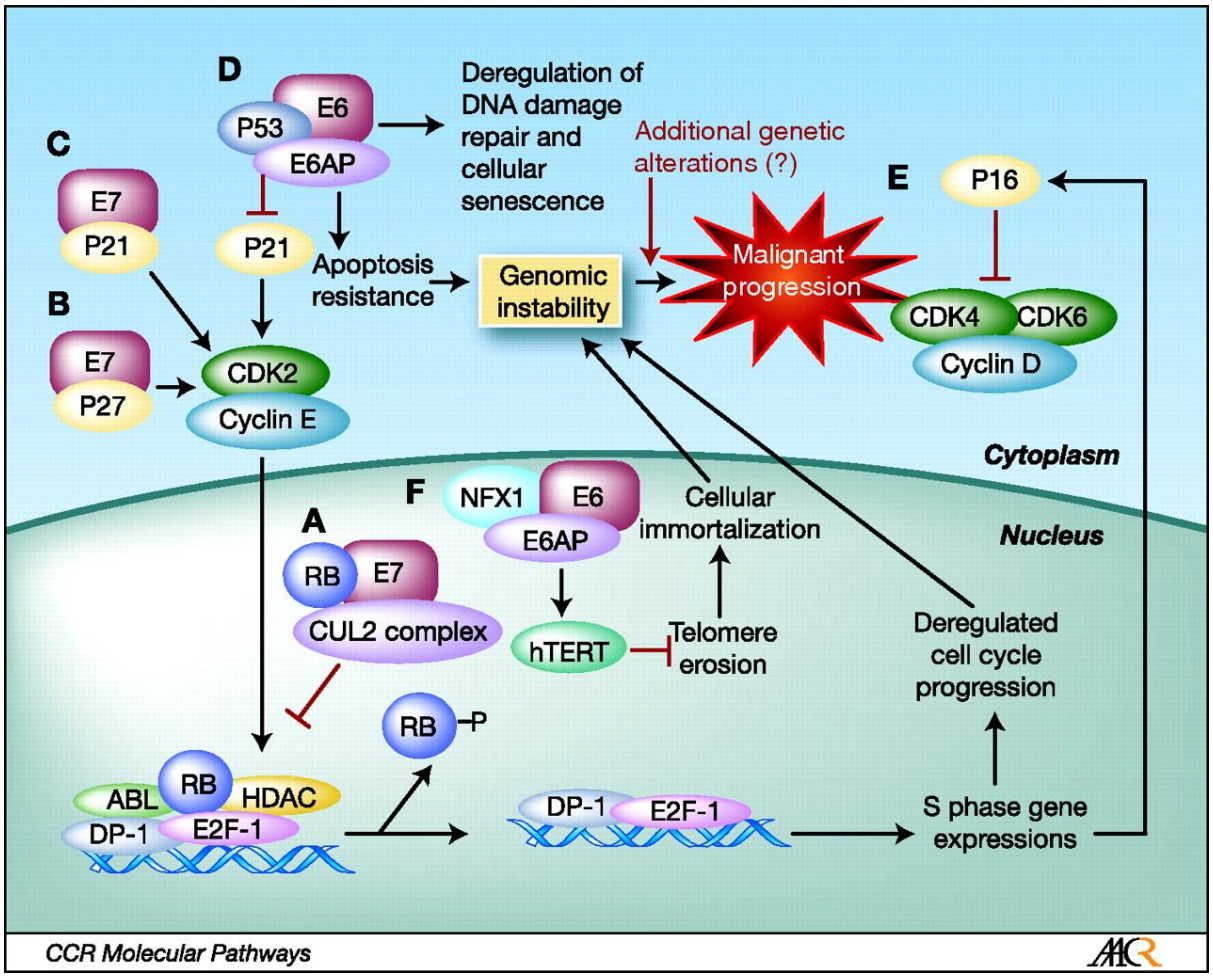


Figure 2.5: Diagram of malignant transformation in keratinocytes caused by the HPV oncoproteins, E6 and E7; clockwise from A, ubiquitination by E7 and the cullin 2 ubiquitin ligase complex leading to pRb degradation B, interaction between E7 and p27Kip1 resulting in inhibition of cell cycle arrest contributing to carcinogenesis; C, interaction between E7 and p21Cip1 resulting in inhibition of cell cycle arrest contributing to carcinogenesis; D, ubiquitination by E6 and ubiquitin ligase E6AP leading to p53 degradation; E, increased expression of p16INK4A by a consequent of feedback loops from the absence of pRb function; and F, degradation of NFX1, a transcriptional repressor of hTERT, by association with E6/E6AP resulting in hTERT activation and cellular immortalization (AACR, 2017).

Table 2.2: Major roles of proteins expressed by high risk HPV (Graham *et al*., 2010; Review).

Protein	Role in the virus life cycle
E1	Genome replication: ATP-dependent DNA helicase.
E2	Genome replication, transcription, segregation, encapsidation. Regulation of cellular gene expression. Cell cycle and apoptosis regulation.
E4	Remodels cytokeratin network. Cell cycle arrest. Virion assembly.
E5	Control of cell growth and differentiation. Immune modulation
E6	Oncoprotein. Inhibits apoptosis and differentiation. Regulates cell shape, polarity, mobility and signalling.
E7	Cell cycle control. Controls centrosome duplication.
L1	Major capsid protein
L2	Minor capsid protein. Recruits L1. Virus assembly.

2.4.2 HPV replication and epithelial differentiation

The replication cycle of HPV is tightly linked to differentiation of the epithelium it infects. HPV may access dividing basal epithelial cells by falling down microabrasions in the epithelium and attaching to cells using common cell surface molecules such as heparin sulphate proteoglycans. α6-integrin may be used as the virus receptor at least in some cases, and virus entry is through either calveolar or clathrin-mediated endocytosis (Smith *et al.*, 2007). Once inside the cell, uncoating occurs and the circular virus genome is transported to the nucleus.

Upon division of the infected cell the nuclear episomal genome is replicated to 20-100 copies and maintained in daughter cells upon segregation through attachment of virus genomes to host chromosomes via the virus replication/ transcription factor E2 following the pattern established for BPV-1 (You *et al.*, 2004). One idea is that during the first phase of infection of basal layer cells only the virus replication proteins E1 and E2 are expressed in order to establish and maintain the low copy number genomes (Ibeanu, 2011). Although the E6 and E7 oncoproteins are also expressed soon after infection, the exact temporal lineage of early events in the virus life cycle is difficult to evaluate. This is because it is still technically very difficult to infect epithelial cells and follow the course of infection with HPV. Infection may persist in the infected stem cell, and its daughter cells, in the basal layer of the epithelium over a long period of time, up to several years.

Persistence is considered the major risk factor underlying cervical tumour progression (Woodman *et al.*, 2007). However, a normal infection requires that division of a basal stem cell sometimes yields a daughter transit amplifying cell with the ability to differentiate to upper epithelia layers. Normally, upon differentiation epithelial cells stop dividing and

commence terminal differentiation. Importantly, in HPV-infected epithelial cells the concerted action of the E6 and E7 oncoproteins and their partner proteins p53 and pRb respectively, reactivates cell division, inhibits apoptosis and abrogates epithelial differentiation (Hamid *et al*., 2009).

Unscheduled division of infected cells in the differentiating epithelial layers gives rise to the physical manifestation of infection as a wart. Reactivated cell division allows further amplification of virus genomes to yield many thousands of copies. Finally, upon differentiation to the granular epithelial layer, the virus capsid proteins L1 and L2 are expressed and encapsidate the newly synthesised virus genomes (Ibeanu, 2011). For release, new virions may rely on mechanical breakage of infected cells sloughed off from the upper later of the epithelium. However, evidence exists that the E4 protein has an active role in priming the infected differentiated cell by disrupting the keratin filamentous network and inhibiting formation of the cornified envelope (Graham, 2006).

Figure 2.6 summarised the above description that is relevant for the anogenital infective HPV. Although the same general scheme may apply to HPV of the β-genus, some important differences do exist. For example, cutaneous HPV E6 and E7 proteins do not appear to operate by the same mechanisms as anogenital HPV in regulating the cell cycle, differentiation and apoptosis of the infected cell (Figure 2.7). The temporal organisation of the virus replication cycle is also different between different HPV types perhaps reflecting differences in sites of infection and in different modes of transmission (Woodman*et al*., 2007).

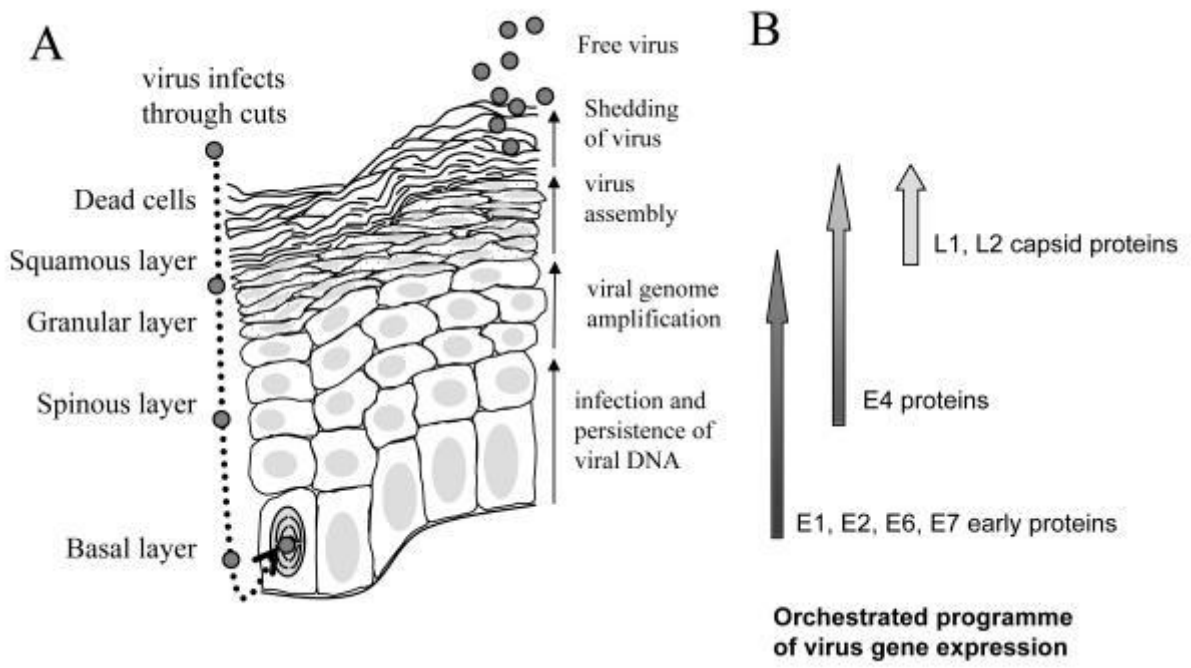


Figure 2.6: The replication cycle of high risk HPV in a differentiating epithelium
(Graham *et al*., 2010)

A. The different epithelial layers are indicated on the left hand side of the diagram. Virus shown as small grey circles. The nucleus of the infected, dividing basal epithelial cell is indicated with curved lines representing condensed chromosomes. All nuclei are shaded in light grey. The granular layer is shown with dotted cytoplasm. The key events in the virus replication cycle are indicated to the right hand side of the diagram of the epithelium.
B. A schematic diagram of the gene expression program of the virus within the infected epithelium. Shading on the arrows represents the quantity of expression of each protein subset during the virus replication cycle.

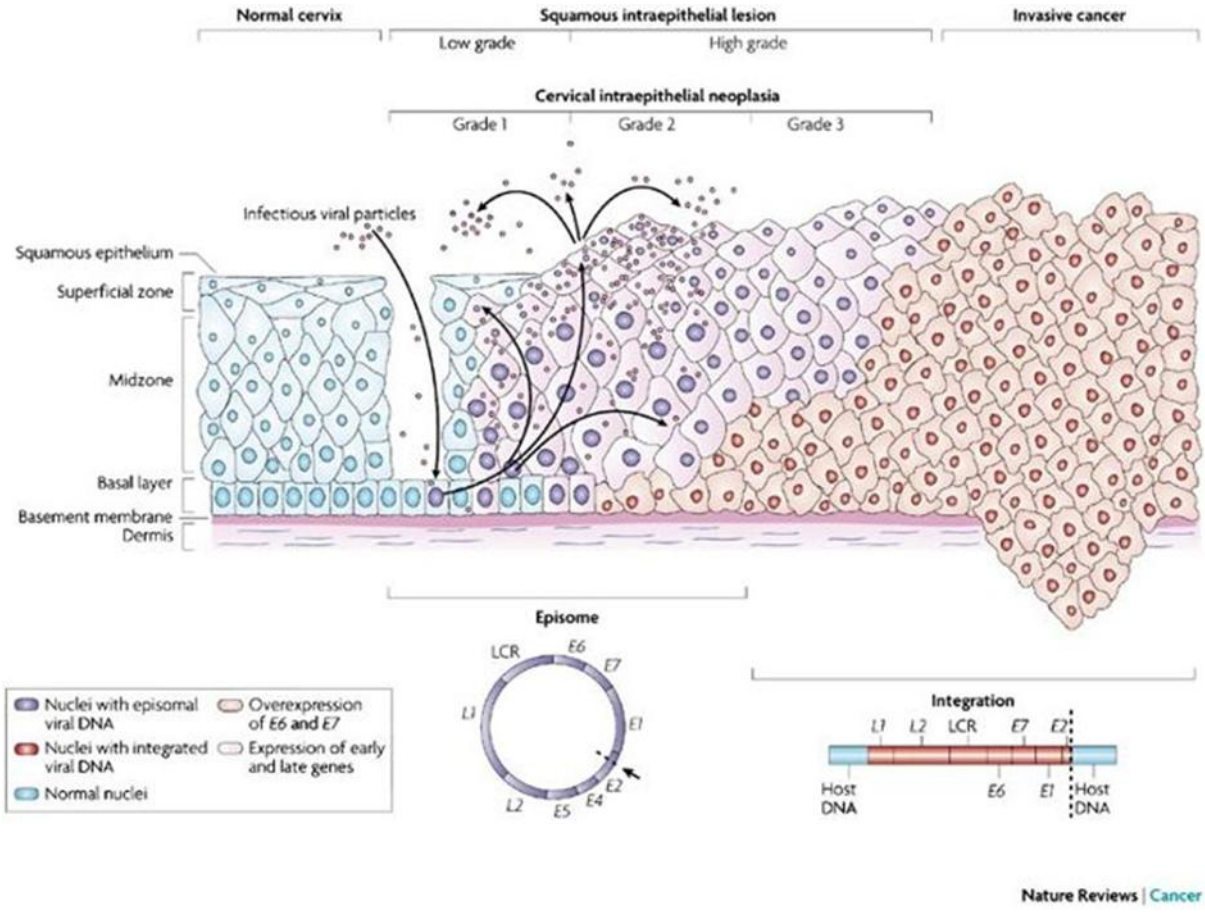
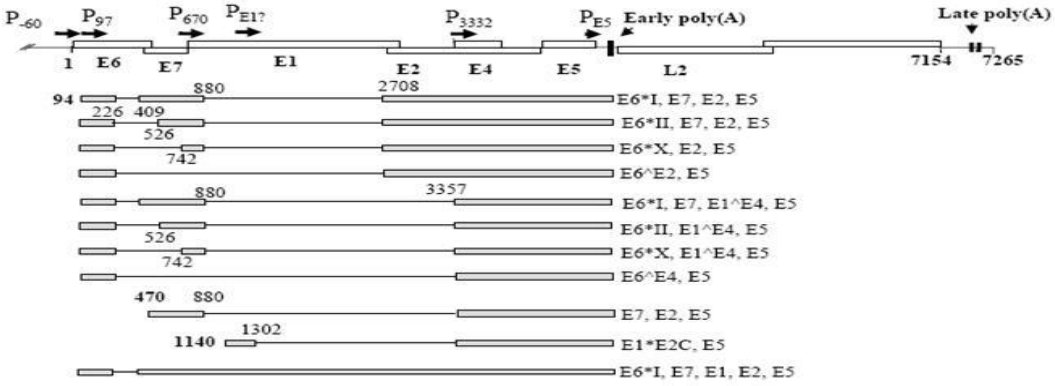


Figure 2.7: HPV-mediated progression to cervical cancer

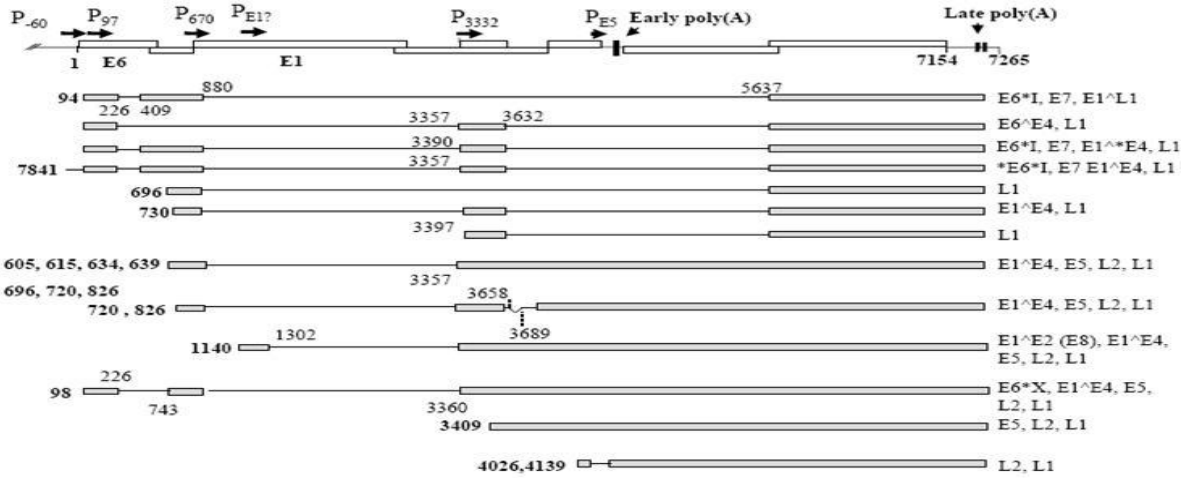
2.5 HPV Gene Expression

2.5.1 Transcription

Transcription of the circular virus genome occurs from only one DNA strand, is initiated from more than one promoter region and is polycistronic, yielding multiple mRNAs with several open reading frames (Figure 2.8). A major problem with RNAs that potentially encode several proteins is that only the first open reading frame in each mRNA will be translated efficiently (Graham, 2006). Expression of multiple mRNAs from the virus genome may be necessary to ensure that each open reading frame is first in at least one mRNA.



HPV16 mRNAs ending at the early polyadenylation site detected in W12 cells



HPV16 mRNAs ending at the late polyadenylation site detected in W12 cells

Figure 2.8: The gene expression profile of HPV16 in W12 cervical epithelial cells
(Zheng and Baker, 2006, Milligan *et al*., 2007)

A. At the top is shown a diagram of the HPV16 genome linearised at the first nucleotide. Open boxes, open reading frames with proteins encoded indicated with bold text. Promoters are indicated with arrows and "P". The early and the two late polyadenylation sites are indicated with arrowheads and vertical bars on the genome map. At the bottom is a diagram of the W12 early mRNAs discovered to date (Zheng and Baker, 2006). Grey boxes, exons; black lines, introns. Numbers in bold indicate transcription start sites. The remaining numbers locate splice donor and acceptor sites. The coding potential of each mRNA is indicated to the right hand side.

B. A diagram of the late mRNAs detected in W12 cells with similar annotation as in part (A) (Milligan *et al*., 2007).

For the α-HPV there are two virus promoters that have been well characterised, the early promoter (P97 for HPV16 and 31, P105 for HPV18) in the virus long control region (LCR) that appears to be constitutively active throughout the virus replication cycle [29-32] and a late differentiation-regulated promoter within the E7 open reading frame (P670 for HPV16, P742 for HPV31). Early promoters have also been characterised for HPV6 and 11. For HPV6 there are three, P90, P270, and P680, with HPV11 initiation sites similarly located (Graham, *et al*., 2010).

The P97 promoter of HPV16 is perhaps the best understood (Figure 2.9). It is controlled by the virus E2 protein but also by an array of cellular transcription factors linking virus gene expression into regulation by the infected cell (Carson and Khan, 2006). There are four binding sites for E2 in the promoter; two in the proximal promoter adjacent to the TATA box, one in the distal promoter region and one upstream of the keratinocyte enhancer. The keratinocyte enhancer acts synergistically with an auxiliary enhancer to specify tissue tropism (Thierry, 2009). In a normal infection, the P97 promoter is transcriptionally repressed by E2 in order that expression levels of the E6 and E7 oncoproteins remain low and normally do not activate tumour progression. Repression seems to involve a combination of E2-mediated steric hindrance of TFIID abrogating TATA binding protein locating its binding site, the TATA box, and binding of cellular transcriptional repressor proteins, such as Sp1, to the virus LCR. It is clear that the LCR binds many cellular transcription activators. Although all their roles are not clear at present, it is expected that their binding pattern must confer keratinocyte specificity to transcription.

Dynamic changes in the profile of the proteins bound and strength of binding occur upon epithelial differentiation and likely mediate some transcriptional changes necessary for

completion of the virus life cycle (Ibeanu, 2011). It is possible that E2 interaction with cellular proteins that bind the LCR might lead to a more repressive promoter conformation or could finely tune repression in response to epithelial differentiation. Truncated E2 isoforms that might act as dominant negative transcription factors leading to repression of P97 have also been characterised for HPV16 and 18 although the mechanism by which these operate is unclear because the transactivation domain of E2 necessary for transcriptional repression is missing from the isoforms (Ammermann*et al*., 2008).

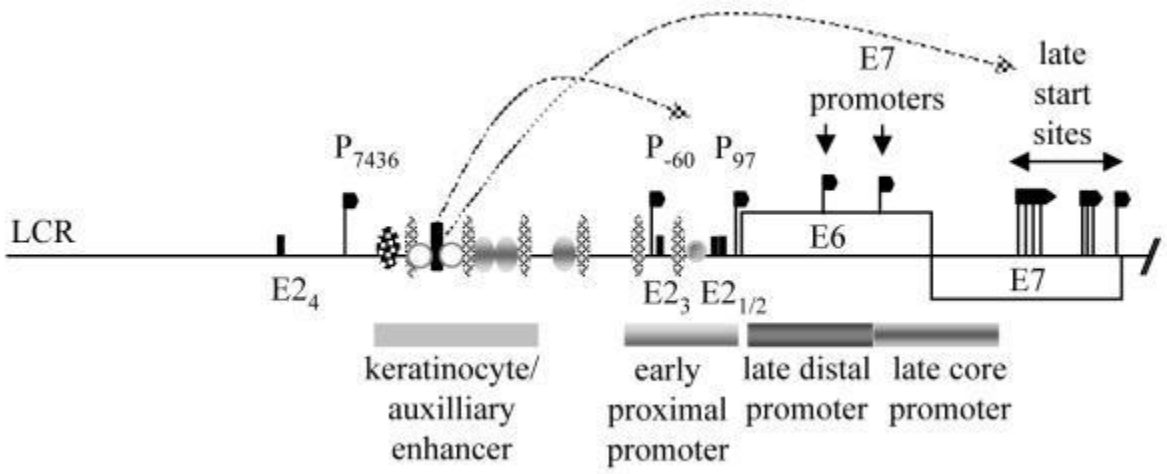


Figure 2.9: Regulation of promoters at the start of the HPV16 genome
(Carson and Khan, 2006)

A schematic diagram of the long control region and the start of the early coding region of the HR HPV genome showing the position of various transcription start sites (black flags), the early proximal promoter region, the keratinocyte/auxiliary enhancer region and the late core and distal promoter regions in the E6/E7 coding region. Data are taken from results with HR HPV16 and 31. LCR, long control region. E2, E2 binding sites indicated by black bars. Ovals and circles indicate approximate regions of transcription factor binding. Only a limited number of binding sites are illustrated. There are very many known binding sites making it impossible to illustrate all transcription factors and their precise binding sites. In addition, most of the transcription factors regulating the E7 promoters and the late promoter have not yet been identified. The box indicates the transcription factors known to bind the HPV16 early region (Carson and Khan, 2006).

The dominant promoter used for transcription initiation late in the virus replication cycle, termed the "late" promoter, is located within the E7 open reading frame and is dependent upon keratinocyte differentiation. For HPV16 and 31, many late promoter-regulated transcription initiation sites have been mapped over a 200 nucleotide region (Milligan *et al*., 2007). For example, HPV31 has over 30 initiation sites stretching from genome position 605 to 779. This complexity in transcription initiation, plus a clear contribution from cis-acting control elements in the LCR, has led to difficulty in mapping cis-acting control elements for the late promoter.

The HPV31 P742 core promoter has been mapped to a 150 nucleotide region upstream of the region of most late transcription initiation sites. Furthermore, two differentiation responsive elements, DRE1 and DRE2 have been delineated in the E6/E7 gene region (Bodily and Meyers, 2005). However, the mode of action of these is not necessarily at the level of control of transcription initiation. One or both elements may regulate stability of certain late mRNAs if they reside in the 5′ untranslated region. It is clear that chromatin remodelling regulates the late promoter in a differentiation stage-specific manner as DNase I hypersensitive sites are uncovered upon differentiation of virus genome-containing keratinocytes (Graham, *et al.*,2010). Interestingly, the keratinocyte enhancer in the long control region contributes to regulation of the late promoter. Further studies are required to examine the relative contributions of the different regulatory regions to differentiation stage-specific transcription of the virus genome.

Apart from the major early and late promoters, the existence of other promoters has been reported in various HPV genomes. It seems likely that each HPV has a similar array of promoters in the early region of the genome to that found for BPV-1 (Zheng and Baker, 2006). Several novel transcription start sites upstream of the P97 promoter have been observed in a number of HPV (Hansen *et al*., 2010). At least one located in the LCR is active

in expression of mRNAs late in infection. A promoter at the start of the E1 open reading frame may also exist at least for HPV16, but this has not been proven definitively (Milligan *et al*., 2007). Several studies have verified the existence of a late promoter at the start of the E4 open reading frame (Milligan *et al*., 2007). This promoter is particularly interesting as it may express the mRNA from which L1 capsid protein is the first open reading frame, thus ensuring efficient translation.

Finally, a promoter in the E5 open reading frame has been demonstrated for HPV6 and 16 that can transcribe mRNAs encoding L2 as a first open reading frame. Regulation of these transcription starts has not yet been elucidated and, like the "late" promoter, may prove complex involving multiple genome regions (Milligan *et al*., 2007).

2.5.2 Post-transcriptional events

Although HPV gene expression is regulated at the level of transcription initiation and in response to epithelial differentiation signals, it is clear that post-transcriptional regulation is also very important. The HPV genome is compact. Polycistronic transcription, overlapping open reading frames, regulated read-through of the early polyadenylation site and alternative splicing ensure maximum coding potential but mean that regulation of RNA processing in the nucleus and translation in the cytoplasm must be key to regulation of virus gene expression. For example, an early observation that L1-encoding mRNAs could be detected in lower epithelial layers where the capsid proteins are not expressed indicated post-transcriptional regulation of virus late gene expression (Graham, 2010). There are multiple possibilities for virus post-transcriptional regulation.

2.5.3 Polyadenylation

Upon transcription, 3′ end formation of virus RNAs may occur either at the early or late polyadenylation sites (Figure 2.10). The early polyadenylation site, as studied for HPV16 and

31, is “weak” meaning that it should be utilised poorly. A consensus AAUAAA polyadenylation signal that binds cleavage and polyadenylation specificity factor (CPSF) is present between the E5 and L2 open reading frames. Co-binding of CPSF with cleavage stimulatory factor (CstF) regulates efficiency of 3′ end formation. Three different G/U-rich regions located downstream of the early cleavage site were found to bind CstF only weakly, and so this could be responsible for inefficient use of HPV early poly(A) sites. The presence of degenerate CPSF binding sites and more than one G/U-rich CstF binding site may also explain the observed heterogeneity in early 3′ end formation (Terhune*et al*., 1999). Clearly, the early polyadenylation site is active in formation of viral early mRNAs. However, its inherent inefficiency may be important late in the virus replication cycle to allow production of late mRNAs that are transcribed through the early polyadenylation region and which terminate within the 5′ portion of the LCR downstream of the L1 open reading frame (Figure 2.10B, Late poly(A)) (Terhune*et al*., 1999; Terhune*et al*., 2001).

Sequences in the HPV16 E4 open reading frame, an upstream polyadenylation element in the early 3′ untranslated region and a polyadenylation element in the first 174 nucleotides of the L2 open reading frame may act to enhance efficiency of early polyadenylation in an epithelial differentiation-specific manner at least in the context of reporter gene assays (Ibeanu, 2011). The L2 element possesses G-rich motifs that bind CstF 64, a component of CstF polyadenylation complex and hnRNP H that may stimulate polyadenylation complex assembly. Elements that repress activity of the HPV31 early polyadenylation site during the virus replication cycle have been mapped within the first 800 nucleotides of the L2 open reading frame but their mechanism of action is unknown (Terhune*et al*., 2001). For HPV16 there are two late polyadenylation sites located in the late 3′ untranslated region just downstream of the L1 open reading frame (Figure 2.10B). Each possesses good consensus

polyadenylation signals and HPV16 mRNAs terminating at each site have been detected in infected cervical epithelial cells (Milligan *et al*., 2007).

2.5.4 Splicing

Although HPV do express some unspliced mRNAs, most HPV mRNAs are the products of constitutive and alternative splicing. Constitutive splicing is where every intron in the primary RNA transcript is removed as transcription progresses along the gene. Alternative splicing is where exon or intron skipping can occur. For HPV, several main splice events can occur. The first is generated by splicing from a splice donor site in the E6 open reading frame and the second from one at the end of the E4 open reading frame each onto a range of splice acceptor sites located in the E6, E7, E4 and L1 regions of the genome. Both splice events occur in some mRNAs (Graham, *et al*., 2010).

Regulation of splicing is complex and occurs through efficient recognition of cis-acting splicing signals and RNA/protein complexes known as spliceosomes that form across the exon-intron junction. Splicing efficiency is regulated by other cis-acting signal sequences within exons, termed exonic sequence enhancers (ESE) or silencers (ESS) or within introns termed intronic sequence enhancers (ISE) or silencers (ISS) (Wang and Burge, 2008). The enhancer elements bind members of the serine-arginine (SR)-rich protein family of which there are 9 classical members and a number of other related factors (Long and Caceres, 2009). Conversely, silencers bind members of the hnRNP protein family (Martinez-Contreras *et al*., 2007). SR and hnRNP proteins antagonise the action of each other and complex combinations of these factors may often be required to regulate splicing (Barash*et al*., 2010).

Splicing regulatory sequences on the HPV genome have been defined through analysis of splicing reporter expression plasmids. In the case of HPV16, an ESE required for efficient use of the splice acceptor site 3358 at the start of the E4 open reading frame and an ESS

regulating efficiency of the splice donor site at the 3′ end of the E4 open reading frame have been demonstrated (Figure 2.10). This ESE binds the smallest SR protein, SRp20. By analogy with the situation for BPV1, SRp20 may control selection of the splice acceptor site required for synthesis of fully processed virus L1 capsid mRNAs. This ESE also controls early polyadenylation site efficiency. This dual role is unsurprising as exonic regulatory sequences are frequent players in defining exons by cross talk with downstream polyadenylation complexes. Proteins that bind, and mechanism of action of the E4 ESS, are unidentified as yet.

ESS are found within the L1 open reading frame and bind hnRNP A1 to inhibit expression of L1 capsid mRNA. The silencing effect may be abrogated by an ESE in the L1 region demonstrating the potential complexity of splicing control on RNA expressed from an intact virus genome (Figure 2.10) (Graham, 2010). Future studies must focus on global control of HPV splicing regulation and charting which mRNAs are expressed in conditions of overexpression and knock down of individual splicing regulatory proteins.

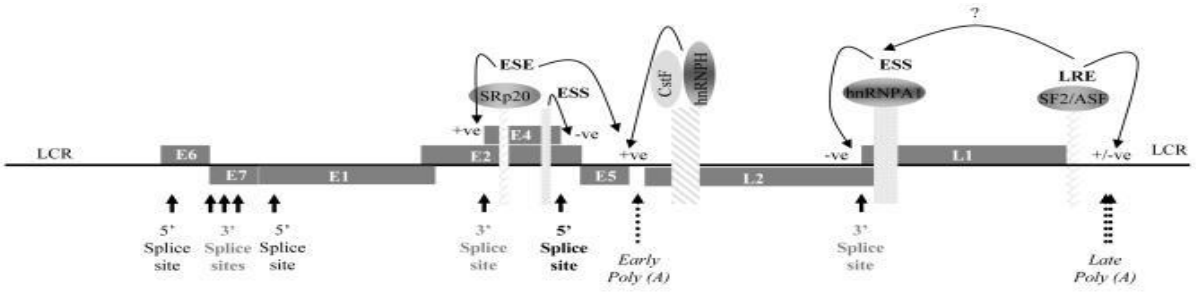


Figure 2.10: HPV16 RNA processing regulation; ESE, ESS and polyadenylation control elements and their binding proteins(Graham *et al*., 2010)

A schematic diagram of the HPV16 genome showing open reading frames (grey rectangles containing gene names), splice donor (5′) and splice acceptor (3′) splice sites (black arrows), polyadenylation sites (dotted arrows). When transcribe to RNA, cis-acting RNA processing regulatory elements (patterned oblongs) and the proteins these bind (annotated ovals) are indicated. Arrowed lines indicate the target and type of regulation of each RNA/protein interaction. LCR, long control region (Graham, 2010).

Importantly, HPV regulate expression of a subset of SR proteins that control viral RNA splicing of viral. Levels of the three smallest SR proteins, SRp20, SC35 and SF2/ASF are specifically increased in the mid to upper layers of HR HPV-infected cervical epithelia (Mole *et al*., 2009). The HPV E2 transcription factor controls expression of these SR proteins during infection. For SF2/ASF at least, the mechanism is by trans-activation of the promoter of its gene (Mole *et al*., 2009b). E2-mediated SF2/ASF upregulation during the late phase of the virus replication cycle is necessary for expression of the virus capsid proteins and therefore necessary for completion of the virus replication cycle (Graham, *et al*., 2010).

2.5.5 Polymorphisms of the HLA region

As the allelic variants of HLA are highly polymorphic, polymorphisms in genes in HLA-class I (HLA-A, HLA-B, HLA-C, HLA-G) and HLA-class II (HLA-DP, HLA-DQ and HLA-DR) have been identified (Mora, et al., 2016). HLA-A polymorphisms relationship was observed between HLA-A polymorphisms and the danger of developing cancer. Generally, without regard to the infectious stage of the HPV, there is a reduced risk of developing invasive cervical cancer (ICC) in patients carrying the A* 0207/0215N and A* 2402 alleles, which may confer a possible protective effect against the neoplasia (Chan, et al., 2005).

HLA-B polymorphisms B* 07 and DQB1* 0302 polymorphisms increased the likelihood of suffering high-grade squamous intraepithelial lesions (HSIL) and low-grade squamous

intraepithelial lesions (LSIL) 8.2 and 5.3 times, respectively (Wang, et al., 2001). It has been proposed that the B* 07 polymorphism is a potential molecular biomarker for a screening test for that carcinoma (Qiu, et al., 2011).

HLA-C polymorphisms: Both killer cell immunoglobulin-like receptors (KIR) and their ligands have been related to the development of CC. In women with ICC, an association of the HLA-C locus with the risk of developing the neoplasia has been suggested, possibly due to the action of certain NK cell ligands, specifically KIR ligands, after verifying the presence of the HLA-Cw ligand in women with ICC and particularly those infected withHPV16 and HPV18 (Martin, et al., 2010).

HLA-DP polymorphisms: The HLA-DP rs3077 and HLA-DP rs9277535 polymorphisms have been proposed as susceptibility markers for CCU as it has been shown that they increase the possibility of contracting the neoplasia in Chinese women. It has been suggested that SNPs may play a role not only in CC but also in the persistence of HPV infection (Jiang, et al., 2013).

HLA-DQ and HLA-DR polymorphisms: One of the first complete studies on the HLA region was carried out on 1,006 Dutch patients with differentiated cervical neoplasias. The 221 and 184 alleles were analyzed for their proximity to the region containing the HLA-DQ and HLA-DR genes. A significant decrease in the frequency of the 221 allele and a strong association of the 184 allele in patients with SCC suggest G51125 and MICA as genetic markers for susceptibility to CC (Zoodsma, et al., 2005). From that report, the HLA-DQ and HLA-DR regions, particularly the HLA-DRB1 and HLA-DQB1 alleles, have been widely studied for their involvement in the development of CC.

HLA-G polymorphisms: The influence of various polymorphisms in the 3' UTR region of the G antigen of major histocompatibility complex I (HLA-G 3'UTR) has been linked to CC. In

Canadian women positive for HPV, the risk factor for developing ICC was significantly enhanced in cases with homozygous genotypes for G* 01:01:02, G* 01:06 and G* 3'UTR 14-bp In/In, whereas the cases with heterozygosity for the wild allele (G* 01:01:01) were significantly associated with a reduced risk of an invasive carcinoma (Ferguson, et al., 2012). It has been reported that the (∗) In/G and (∗) Del/G haplotypes are associated with increased and decreased risk of HSIL and ICC (Silva, et al., 2013). The (∗) Del/G haplotype has also been linked to a lower probability of developing ICC in Italian patients with differing histological degrees of CC and positive for HPV.

Very recently, the +3142C/C polymorphism was associated with an increase in the risk factor for SCC in Taiwanese women and, in addition, its frequency is high in the subgroup of women positive for HPV16 (Yang, et al., 2014).

2.5.6 Challenges of HPV diagnosis in cervical smears

A major issue of high relevance for treatment of HPV-related disease in the clinic is accurate diagnosis of severity of disease (Woodman *et al*., 2007). In particular for cervical lesions, current approaches use cytological assessment of Pap smear material which is low sensitivity and subjective in nature, yielding false positive and negative results. Detection of HPV DNA by PCR is exquisitely sensitive and specific and is used in certain settings. However, again results can prove misleading as DNA detection can indicate the presence of viruses but not whether the virus is actively expressing the proteins that cause disease (Graham, *et al*., 2010).

Understanding virus gene expression patterns in disease and during disease progression will aid development of novel accurate diagnostic tests. Improved accuracy in diagnosis will inform treatment regimens and save money in terms of patient treatment and may alleviate anxiety in patients diagnosed with HPV infection but whose disease may not progress to

tumour. Improvements in diagnosis will arise from identification of hallmarks of active virus infection, present in low grade cervical lesions, and specific biomarkers of high grade disease. Analysis of HPV protein is therefore necessary to overcome this challenge. mRNA profiling could also be used to determine if virus-infected tissue is expressing virus proteins and which proteins are being expressed. mRNA profiling gives a depiction of viral activity and therein a potential handle on the clinical significance of the infection. Currently, quantitative PCR to detect E6 and E7 mRNA expression has recently been developed and is prove to be one of the most promising tests to detect high grade lesions. For example, "Pre-tect HPV proofer" (Norchip) detects the HR-HPV E6 and E7 mRNAs and may specifically detect persistent infections. Completion of HPV transcription profiles at different stages of cervical disease may prove essential in developing novel diagnostic tests that distinguish between transient and persistent infection (Graham, 2010; Schmitt *et al.*, 2010).

2.5.7 E6 and E7 immune modulation

In addition to the combined expression of E6 and E7 in high-risk HPV types greatly enhancing cellular transformation, E6 and E7 also modulate molecules involved in the innate and adaptive immune responses, potentially enabling HPV to evade immune surveillance, especially in the early stages of replication (Ibeanu, 2011).

E6 inhibits the ability of interferon regulatory factor 3 to induce the activation of interferon beta, thus abrogating this early response of the innate immune system to viral infection. E7 also binds to interferon regulating factor 1 to prevent activation of interferons alpha and beta. Likewise, the inhibition of toll like receptor 9 (TLR9) and subsequent failure of cytokine production by the expression of E6 and E7 has been demonstrated in tissue culture studies (Martinez-Lagunas*et al.*, 2010).

Cervical cancer cells show an abnormal response to the action of transforming growth factors (TGFs), which typically act to prevent uncontrolled epithelial cell growth. Downregulation of tumor necrosis factor (TNF) alpha expression, as well as resistance to the effects of TNF alpha and beta, have been observed in cervical cancer cells. While not entirely understood, it is thought that E7 inhibition of CKIs p15, p21 and p27 is partly responsible for the absence of effects of TGFbeta in infected epithelial cells (Martinez-Lagunas *et al*., 2010).

E6 modulates the contact between epithelial cells and antigen presenting cells (Langerhans cells) in the epidermis by affecting the cell surface protein, E-cadherin. Diminished Langerhans cell numbers and reduced E-cadherin are recognized features of HPV infection. Epithelial cells normally secrete cytokines to induce chemotaxis, which leads to the repopulation of Langerhan's cell precursors (Ghittoni*et al*., 2010). This chemotactic activity is mediated through the macrophage inflammatory protein, MIP-3alpha. MIP-3alpha expression is inhibited in cells that show evidence of E6 and E7 expression with the resultant impairment of antigen presentation (Ibeanu, 2011).

In addition to the above E6 and E7-mediated immune effects, the HPV replication cycle appears to confer certain survival advantages that allow for HPV infections to persist for long periods in certain individuals. The intraepithelial sequestration of the replication cycle allows for the absence of a blood borne phase (Yugawa and Kiyono, 2009). This absence of viremia allows viral replication to proceed somewhat covertly without triggering the immune system. The release of viral particles from the terminal (apical or top layer) epithelial cells, as well as non-lytic replication, avoids provocation of anti-inflammatory molecules.

Asymptomatic HPV infections are the norm in immunocompetent women, even in those with highrisk HPV infections, because most (younger) women clear their infections within 12 months (zurHausen*et al*., 2000). This observation is obviously confounded by the fact that it

is quite possible for the same patient to acquire different HPV types going forward in time, such that the contribution of one or another HPV type to the clinical picture of persistence is hard to separate. The reason for persistence and progression of lesions in a minority of patients is not well understood.

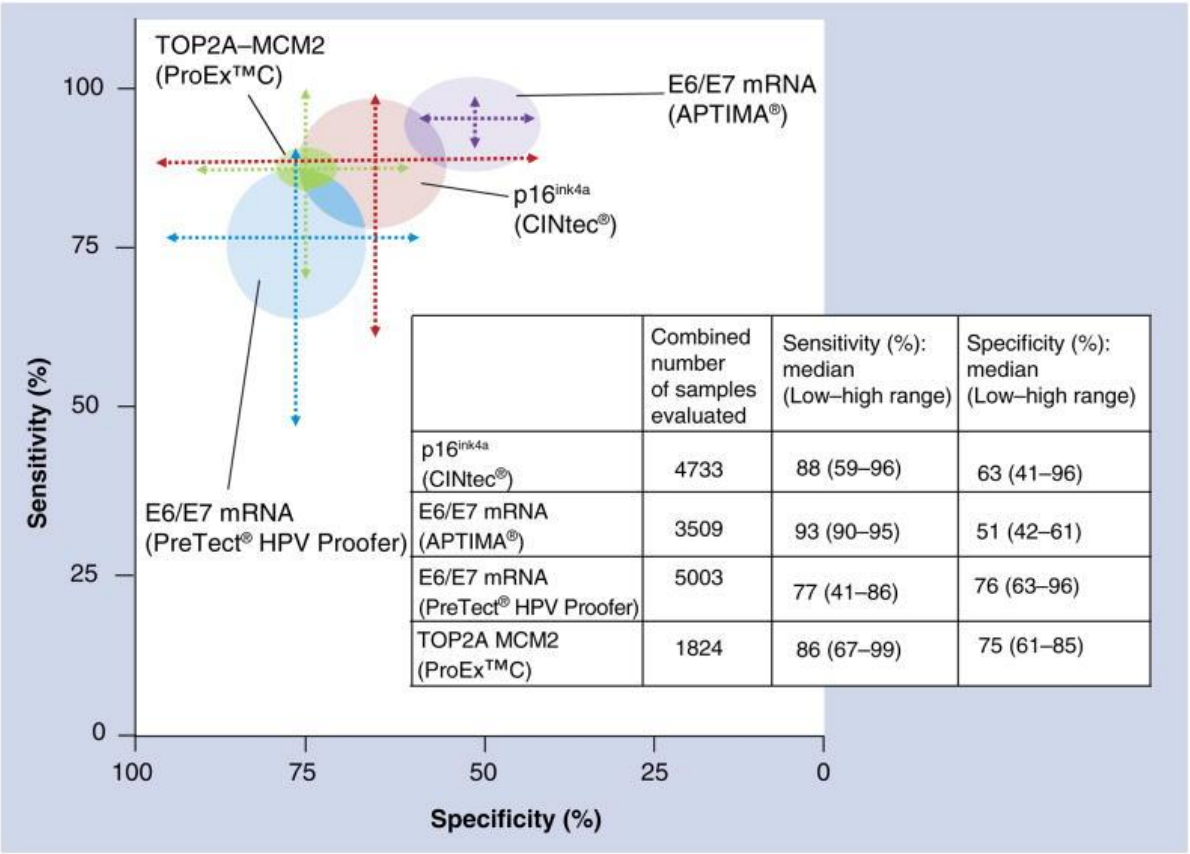
The average lag time from initial HPV infection to carcinogenesis is 10–20 years (zurHausen*et al*., 2000). The integration of viral DNA into host cells is a typical event in carcinogenesis, but is thought to occur sporadically. This suggests that there may be other, yet unknown, environmental and genetic factors that influence the propensity of HPV to remain latent, regress or progress with time (Ibeanu, 2011).

2.5.8 p16INK4a/Ki67 markers

p16 (also known as cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1 and as several other synonyms), is a tumor suppressor protein, that in humans is encoded by the CDKN2A gene. On chromosome 9p21, the INK4a locus encodes both p16 and p14ARF proteins by alternative splicing, in which exon 1alpha is used by p16 and exon 1beta is used by p14ARF. Although the role of p14ARF as a true tumor suppressor has yet to be further characterized, p16 fulfills all the criteria for a bona fide tumor-suppressor gene, even judged with the strictest measures (Ai *et al*., 2003). The human p16 protein contains 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin-dependent kinase 4. The tumor suppressor function of p16 is attributed to its ability to inhibit the catalytic activity of the cyclin-dependent kinase 4–6/cyclin D complex that is required for phosphorylation of retinoblastoma protein (Stone *et al*., 1995; Ai *et al*., 2003).

The E2F family of transcription factors is essential for cell cycle progression, and their transcriptional activity is inhibited through the specific binding to retinoblastoma protein b. This retinoblastoma protein/E2F complex will release E2F transcriptional factors upon phosphorylation of retinoblastoma protein by cyclin dependent kinase 4–6/cyclin D complex and promote cell cycle progression past the G1/S checkpoint (Stone *et al*., 1995). By binding to cyclin-dependent kinases 4 and 6 and inhibiting retinoblastoma protein phosphorylation, p16 prevents the formation of cyclin-dependent kinases 4–6/cyclin D stimulatory complex and promotes the formation of retinoblastoma protein/E2F repressive complex, with the end result of blockage of cell cycle progression at the G1/S checkpoint (Ai *et al*., 2003).

Immunocytochemical staining for overexpression of the cell-cycle regulatory protein p16INK4a (p16 cytology) has been shown to be an efficient approach to triage to colposcopy women who have epithelial cell abnormalities categorized as atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL) (Samarawardana*et al*., 2010). In the majority of the studies, p16 cytology was found to provide sensitivity rates for the detection of underlying high-grade cervical intraepithelial neoplasia (CIN of grade 2 or higher, CIN2+), which were similar or slightly lower compared with the sensitivity of testing for the presence of human papillomavirus (HPV) infections (Schmidt*et al*., 2011). At the same time, specificity of p16 cytology-based testing was found to be substantially higher than the specificity of HPV testing in all studies (Figure 2.10). This effect was even higher in women aged younger than 30 years because of the high prevalence rates of HPV infections in the younger age groups (Samarawardana*et al*., 2010). As both sensitivity and specificity are relevant metrics of the performance of tests used for the triage of equivocal or mildly abnormal Pap cytology results, it is important to implement triage algorithms that provide the highest level of specificity without sacrificing sensitivity (Schiffman*et al*., 2007).



	Combined number of samples evaluated	Sensitivity (%): median (Low–high range)	Specificity (%): median (Low–high range)
p16ink4a (CINtec®)	4733	88 (59–96)	63 (41–96)
E6/E7 mRNA (APTIMA®)	3509	93 (90–95)	51 (42–61)
E6/E7 mRNA (PreTect® HPV Proofer)	5003	77 (41–86)	76 (63–96)
TOP2A MCM2 (ProEx™C)	1824	86 (67–99)	75 (61–85)

Figure 2.11: Graphical representation of the range of estimates of sensitivity and specificity of studies evaluating commercially available biomarkers at the cervical intraepithelial neoplasia grade 2+ threshold (Saharabudde, *et al.*, 2011)

The overexpression of p16 in cervical dysplasia has been shown to be associated with the transforming activity of the E7 oncoprotein of high-risk HPV types and can be regarded as a surrogate marker of the E7-mediated inactivation of the tumor-suppressor function of the retinoblastoma protein (pRb). There is experimental evidence that p16 overexpression is induced by the abrogation of a negative feedback mechanism that is mediated by functional pRb at the transcriptional level under normal physiological conditions. Functional inactivation of pRb, though, may lead only to genetic instability and, thus, malignant transformation when it occurs in DNA replication-competent cells. Therefore, simultaneous detection of p16 overexpression and expression of the proliferation marker Ki-67 within the same cervical epithelial cell should indicate deregulation of the cell cycle (Samarawardana*et al*., 2010). Because under normal physiological conditions, the simultaneous expression of a protein with tumor-suppressive function (ie, p16) and a proliferation marker (such as Ki-67) should mutually exclude each other, it may be possible to use the immunocytochemical detection of p16/Ki-67 coexpression to identify cells with deregulated cell cycle in cervical cytology specimens, independent from morphology-based interpretation parameters. The presence of 1 or more double-immunoreactive cells may be used as an indicator of underlying CIN, especially of higher grades (CIN2+) (Schmidt *et al*., 2011) as in Figure 2.12.

Luttmer*et al*. (2016) reported that women who test positive for a high-risk type of the human papillomavirus (HPV) require triage testing to identify those women with cervical intraepithelial neoplasia grade 3 or cancer (≥CIN3). Although Pap cytology is considered an attractive triage test, its applicability is hampered by its subjective nature.

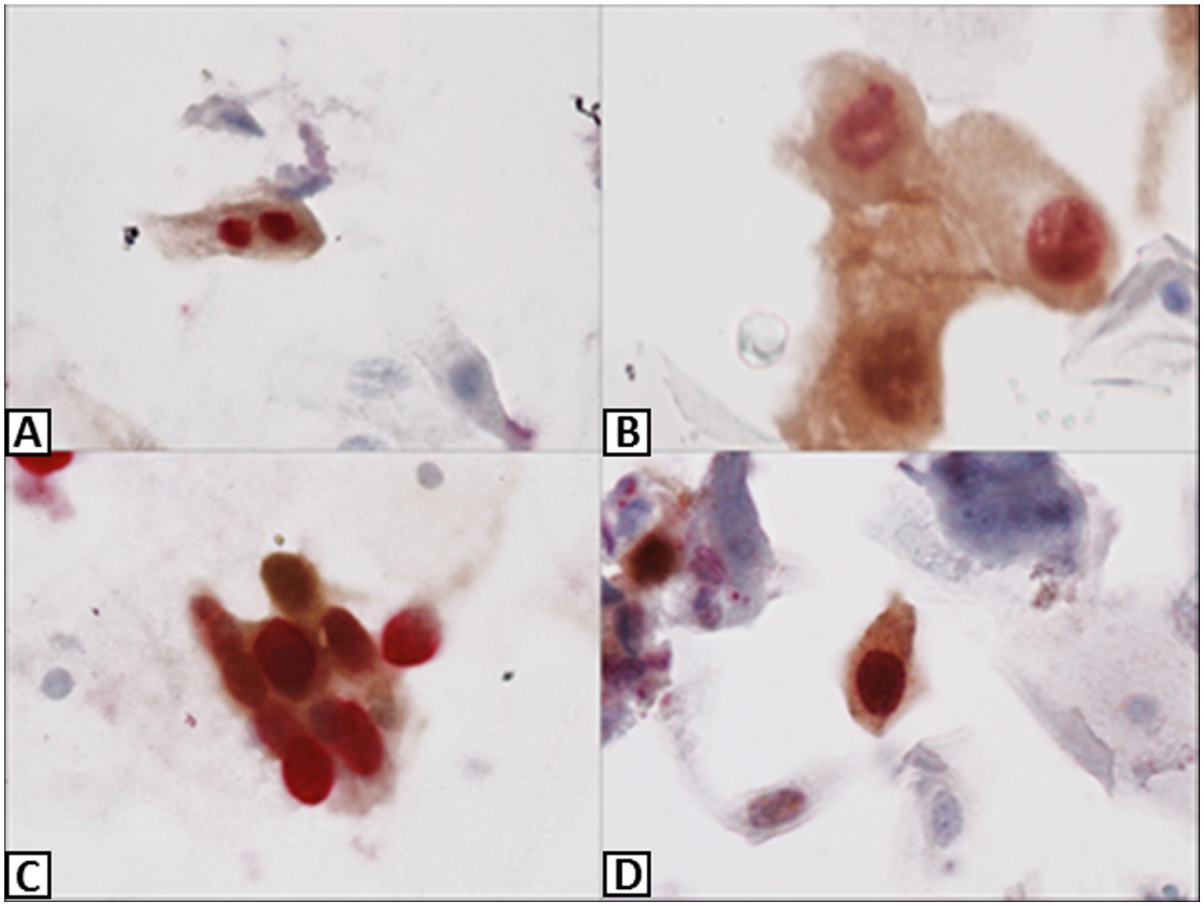


Figure 2.12: Examples of positive tests for Ki-67 (red) and p16 (brown). (A) and (D), Mag x200; (B) and (C). Mag x400 (Schmidt *et al.*, 2011)

2.5.9 TOP2A-MCM2

A biomarker cocktail containing antibodies against minichromosome maintenance protein 2 (MCM2) and topoisomerase IIA (TOP2A) has been reported as a potential diagnostic adjunct for high grade lesions. During DNA replication, MCM2 functions by loading the complex onto DNA before replication and unwinding the DNA through helicase activity to permit DNA synthesis. Topoisomerase IIA is responsible for the enzymatic unlinking of DNA strands during replication (Yang, *et al*., 2013).

Topoisomerase IIA and MCM2 work together in the regulation of DNA replication during S-phase and are overexpressed when S-phase cell cycle induction is aberrant. During the transcriptional activation of the aberrant cell cycle, levels of MCM2 and TOP2A increase in proliferating cells. They have been shown to be overexpressed in various dysplastic and malignant processes, including cervical neoplasia related to high-risk HPV. Any single application of MCM2 or TOP2A will leave out some squamous epithelial lesions, so it is generally approved to apply the cocktail of MCM2 and TOP2A (ProExC) by immunostaining in CIN diagnosis (Yang, *et al*., 2013). See figure 2.13:

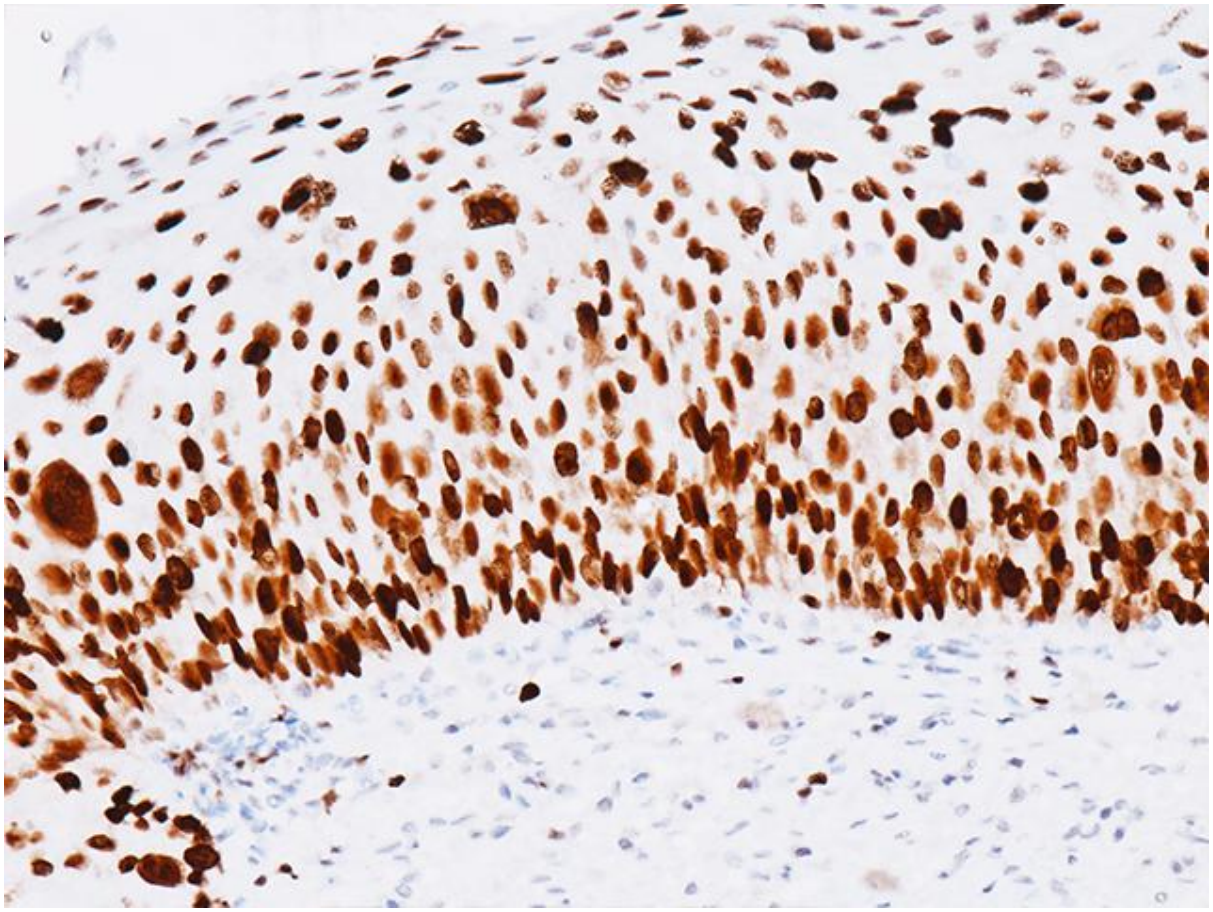


Figure 2.13: Photomicrograph of histological section of the cervix showing positive dual MCM2 and TOP2A immunohistochemical staining. Mag x200 (Yang *et al*., 2013)

2.5.10 p21 protein

Higher eukaryotes have evolved multiple checkpoint mechanisms to monitor and respond to cellular perturbations, halting cellular progression until errors are fixed or the environment becomes permissible to the faithful transmission of genetic material (Bartek and Lukas, 2007). Perturbations in checkpoint mechanisms are detrimental to the integrity of the genome, promote cancer development (Nakanishi *et al*., 2006) and significantly affect the efficacy of anticancer treatment (Eastman, 2004). The tumour suppressor protein p53 mediates the DNA damage-induced checkpoint through the transactivation of various growth inhibitory or apoptotic genes. Among these, the small 165 amino acid protein p21 (also known as p21WAF1/Cip1) mediates p53-dependent G1 growth arrest (Deng *et al*., 1995; Brugarolas*et al*., 1995). Earlier studies supported the view that p21 suppresses tumours by promoting cell cycle arrest in response to various stimuli. Additionally, substantial evidence from biochemical and genetic studies indicates that p21 acts as a master effector of multiple tumour suppressor pathways for promoting anti-proliferative activities that are independent of the classical p53 tumour suppressor pathway (Figure 2.14).

Despite its profound role in halting cellular proliferation and its ability to promote differentiation and cellular senescence, recent studies suggest that, under certain conditions, p21 can promote cellular proliferation and oncogenicity. Consequently, p21 is often misregulated in human cancers, but its expression, depending on the cellular context and circumstances, suggests that it can act as a tumour suppressor or as an oncogene (Table 2.3).

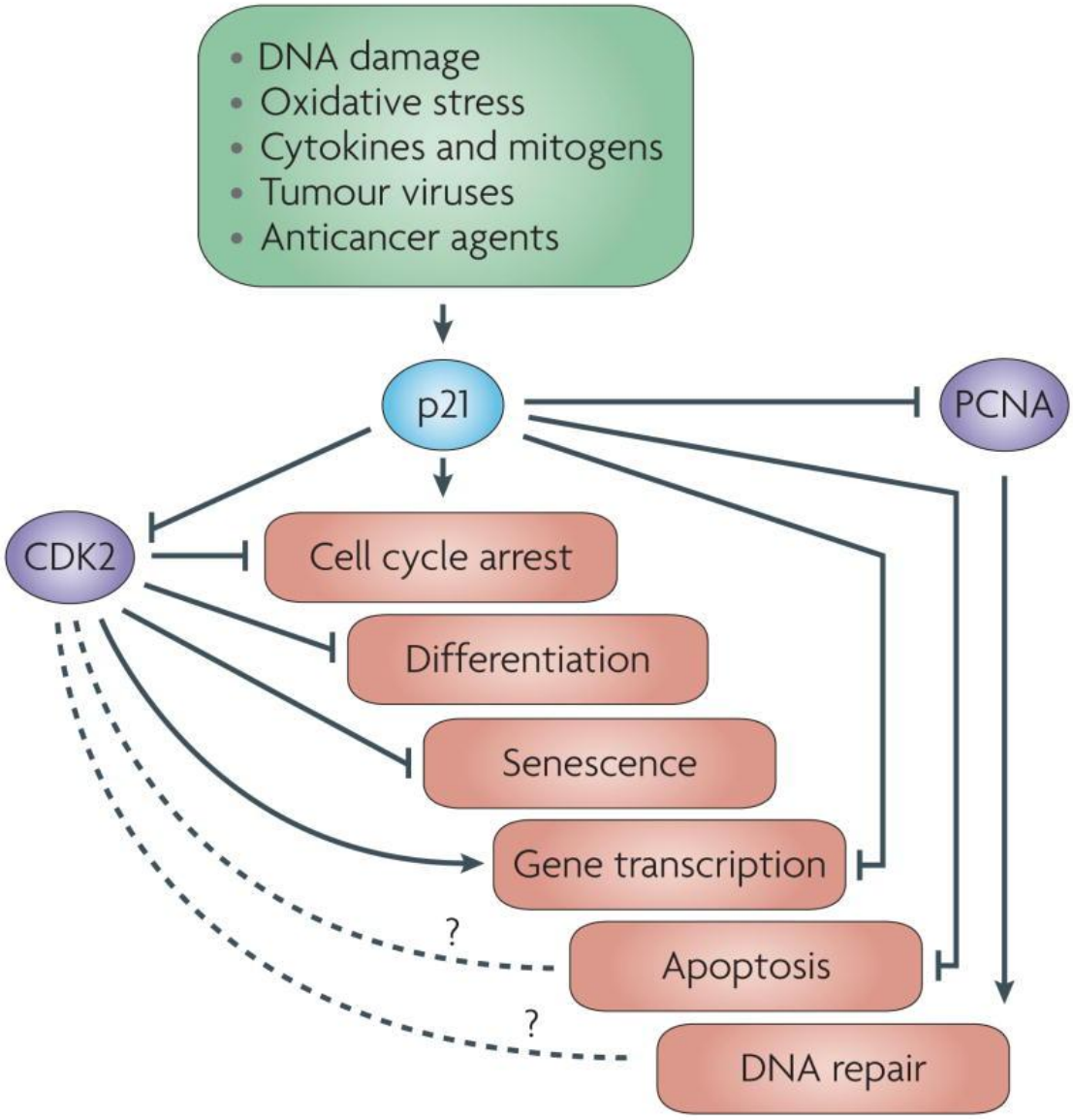


Figure 2.14: The central role of p21 in sensing and responding to a plethora of stimuli

p21 responds to a variety of stimuli to promote growth-inhibitory activities that depend primarily on its ability to inhibit the kinase activity of cyclin-dependent kinase 2 (CDK2). p21-induced cell cycle arrest also depends on its ability to inhibit CDK1. p21 can inhibit cellular proliferation independent of CDK2 inhibition by inhibiting proliferating cell nuclear antigen (PCNA), which is required for S phase progression. Some of the anti-proliferative activities of p21 rely on its multiple protein–protein interactions and its ability to regulate gene transcription. The various physiological responses triggered by p21 are interconnected. For example, cell cycle arrest induced by p21 promotes DNA repair by allowing sufficient time for the damaged DNA to be repaired before it is passed to daughter cells and is a major route by which p21 exerts its anti-apoptotic activities. Similarly, the ability of p21 to regulate gene expression is important in promoting cellular senescence. The effect of p21 on gene transcription is generally inhibitory, but p21 can also activate gene transcription under certain conditions (Abbas and Dutta, 2009).

Table 2.3: p21 deregulation in human cancer (Abbas and Dutta, 2009)

Tissue or cancer	Gene interaction or association	Description of association	Localization
Colorectal cancer	*TP53*	p21 downregulation associates with p53 detection and the development of metastasis and poor survival	Not known
	TP53	p21 downregulation inversely correlates with high microsatillite instability irrespectively of the p53 status	Not known
	None observed	Decreased p21 expression in dysplastic ACFs and adenomas; decreased p21 associated with lymph node and liver metastasis, and poor survival	Not known
	KLF4	*CDKN*1*A* transcripts are downregulated and levels correlate with higher reductions in *KLF4*expression	Not known
Tonsillar carcinoma	HPV	p21 overexpression strongly associates HPV-positive tonsillar SCC with favourable prognosis	Not known
Gastric cancer	*TP53* and *TGFB1*	Those with p21-positive and p53-negative cancers had significantly higher survival curves; all p21- and p53-positive cases were TGFβ1 positive	Not known
Breast cancer	None observed	C94T of *CDKN1A* (Arg → Trp) with inability of p21 to inhibit CDK activity but intact ability to bind PCNA and promote CDK–cyclin association	Not known
Breast, gastric and ovarian cancers	*TP53*	Loss of p21 expression along with increased p53 detection associated with poor prognosis and decreased overall survival	Not known
Oesophageal and oral cancer	None observed	Polymorphism in codon 149 resulting in Asp to Gly substitution	Not known
NSCLC	None observed	Reduced p21 expression in stage III compared with stage I or II	Not known
Cervical adenocarcinoma	None observed	p21 expression correlated with favourable prognosis	Not known

Tissue or cancer	Gene interaction or association	Description of association	Localization
Pancreatic cancer	None observed	p21 is overexpressed in a subset of intraepithelial neoplasia	Not known
Laryngeal and oral carcinoma	None observed	p21 expression correlated with longer overall survival	Not known
Bladder carcinoma	None observed	p21 is a positive marker for invasive cancers, but is a negative prognostic marker in superficial cancers	Not known
Breast cancer	*CCNB1, TP53*	High cytoplasmic p21 levels were associated with high p53 and cyclin B and were significant predictors of poor prognosis	Cytoplasmic
	IKKB	Increased total and cytoplasmic p21 expression was observed in primary cancer and was associated with the expression of IKKβ	Cytoplasmic
	ERBB2	Positive correlation of ERBB2 expression with phosphorylated Akt and cytoplasmic p21; together these were associated with poor prognosis	Cytoplasmic
HCC	HCV	Increased p21 expression correlates significantly with incidence in patients with HCV-associated chronic liver disease; cytoplasmic p21 associated with HCCs, especially in moderately and poorly differentiated HCCs	Cytoplasmic
MM	*TP53*	Nuclear localization of p21 correlates with severity, with PCNA expression and p53 detection, and with poor survival	Nuclear
AML	None observed	High levels of p21 are observed and are associated with poor survival	Not known
Gliomas	*RB1, PCNA*	Overexpression of p21 in 50% of the cases, most notably in higher grades; p21 expression is an indicator of shortened disease-free survival and correlated loosely with PCNA and RB expression	Not known
	None observed	Increased expression of p21 in various types of brain tumours	Not known

Tissue or cancer	Gene interaction or association	Description of association	Localization
Prostate cancer	None observed	p21 overexpression associates with worst clinical outcome before and after androgen deprivation therapy	Not known
Cervical carcinoma	None observed	Increased p21 expression significantly correlated with advanced stage	Not known
Ovarian cancer	None observed	Increased p21 associated with incidence, recurrence and metastasis	Not known
Oesophageal SCC	None observed	p21 is overexpressed; its expression associates with worst overall survival	Not known
Soft tissue sarcomas	None observed	Frequent p21 overexpression	Not known

ACF, aberrant crypt focus; AML, acute myeloid leukaemia; CDK, cylin-dependent kinase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus, HPV, human papilloma virus; IKKβ, inhibitor of nuclear factor-κB kinase-β; KLF4, Krüppel-like factor 4; MM, multiple myeloma; NSCLC, non-small-cell lung carcinoma; PCNA, proliferating cell nuclear antigen; RB, retinoblastoma; SCC, squamous cell carcinoma; TGFB1, transforming growth factor-β1.

p21 mediates its various biological activities primarily by binding to and inhibiting the kinase activity of the cyclin-dependent kinases (CDKs) CDK2 and CDK1 (also known as CDC2) leading to growth arrest at specific stages in the cell cycle (Figure 2.15). In addition, by binding to proliferating cell nuclear antigen (PCNA), p21 interferes with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication and modulating various PCNA-dependent DNA repair processes.

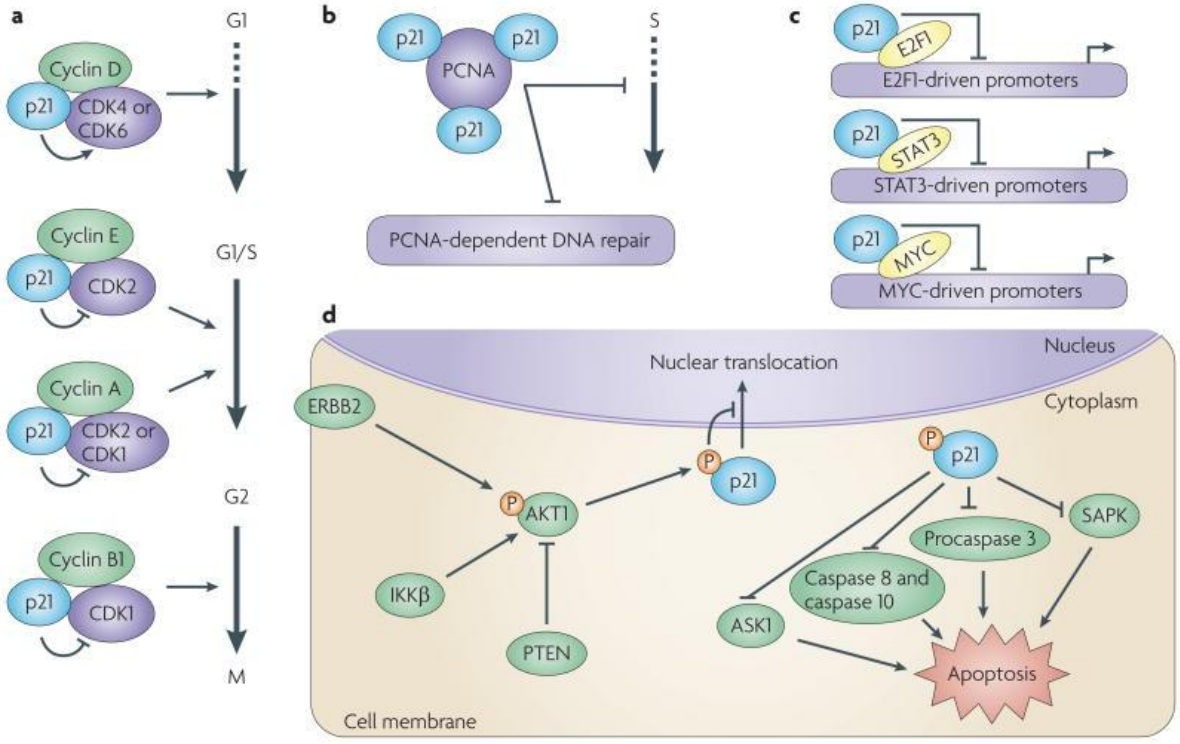


Figure 2.15: The molecular basis of p21 function in cancer

The figure shows activities of p21 in the nucleus and cytoplasm. a | Under certain conditions, p21 promotes the kinase activity of cyclin-dependent kinase 4 (CDK4) or CDK6 in complex with cyclin D, thus promoting progression through G1. p21 inhibits CDK2–cyclin E, with the consequent inhibition of CDK2-dependent phosphorylation of RB and the sequestration of E2F1, thus inhibiting E2F1-dependent gene transcription and progression into and through S phase. p21 also inhibits the kinase activity of CDK2–cyclin A and CDK1–cyclin A, which are required for progression through S phase and into G2 respectively. Additionally, p21 inhibits the kinase activity of CDK1–cyclin B1, thus inhibiting progression through G2 and G2/M. b | Through its carboxyl-terminal domain, p21 binds to and inhibits proliferative cell nuclear antigen (PCNA), thereby inhibiting processive DNA synthesis and modulating PCNA-dependent DNA repair pathways. c | p21 can inhibit the transcriptional activity of the transcription factors E2F1, STAT3 (signal transducer and activator of transcription 3) and MYC through direct binding and inhibition of their transactivation activity. This accounts for some of the anti-apoptotic effects of p21, which may contribute to its oncogenic activity. d | p21 phosphorylation at Thr145 by activated AKT1 (also known as PKB) downstream of ERBB2 (a member of the epidermal growth factor receptor family of receptor tyrosine kinases) or IKKβ (inhibitor of nuclear factor-κB kinase-β) signalling prevents the nuclear translocation of p21. Cytoplasmic p21 exhibits anti-apoptotic activity owing to the inhibition of proteins involved in apoptosis. Whether the phosphorylation of p21 by AKT1 only functions to retain p21 in the cytoplasm or is also required for its cytoplasmic activities is not clear. ASK1, apoptosis signal-regulating kinase 1, also known as MAP3K5; SAPK, stress-activated protein kinase (Abbas and Dutta, 2009).

2.5.11 p27 Protein

Cell cycle progression is driven by cyclins and their associated cyclin-dependent kinases (CDK). p27 was discovered as an inhibitor of cyclin E-CDK2 (Koff, 1993), but has since been shown to play dual roles to both promote and inhibit cell cycle progression. In G0, p27 translation and protein stability are maximal as it binds and inactivates nuclear cyclin E-CDK2. In early G1, p27 also promotes assembly and nuclear import of d-type cyclin-CDKs (Larrea, 2009). The progressive decrease of p27 in G1 permits cyclin E-CDK2 and cyclin A-CDK2 to activate the G1-S transition. Mice lacking p27 display multi-organ hyperplasia, increased body size, and susceptibility to carcinogen-induced tumors, suggesting that p27 acts as a tumor suppressor to control both tissue expansion and cell proliferation (Kiyokawa, 1996; Wander *et al*., 2010).

p27 is regulated at transcriptional, translational, and posttranslational levels. p27 mRNA levels usually show little cell cycle periodicity, but may be repressed in a phosphoinositide 3-kinase (PI3K)/AKT–dependent manner. In normal cells, p27 protein levels are largely controlled by ubiquitin-dependent proteolysis. In early G1, mitogens promote p27 phosphorylation at serine 10 (S10) to facilitate nuclear export (12); this simultaneously relieves cyclin E-CDK2 inhibition and permits Kip1 ubiquitylation-promoting complex (KPC)–mediated proteolysis of cytoplasmic p27 (Kamura, 2004). Tyrosine phosphorylation of p27 by BCR-ABL (Y74, Y88, Y89) or Src family kinases (Y88 and Y74) impairs the CDK2 inhibitory action of p27. This impairment facilitates subsequent cyclin E-CDK2–dependent T187 phosphorylation of p27, which, in turn, allows it to be targeted by SCFSKP2 for degradation (see Fig. 1). Tyrosine phosphorylation of p27 is also required for the catalytic activation of p27-cyclin D1-CDK4 complexes (Wander *et al*., 2010).

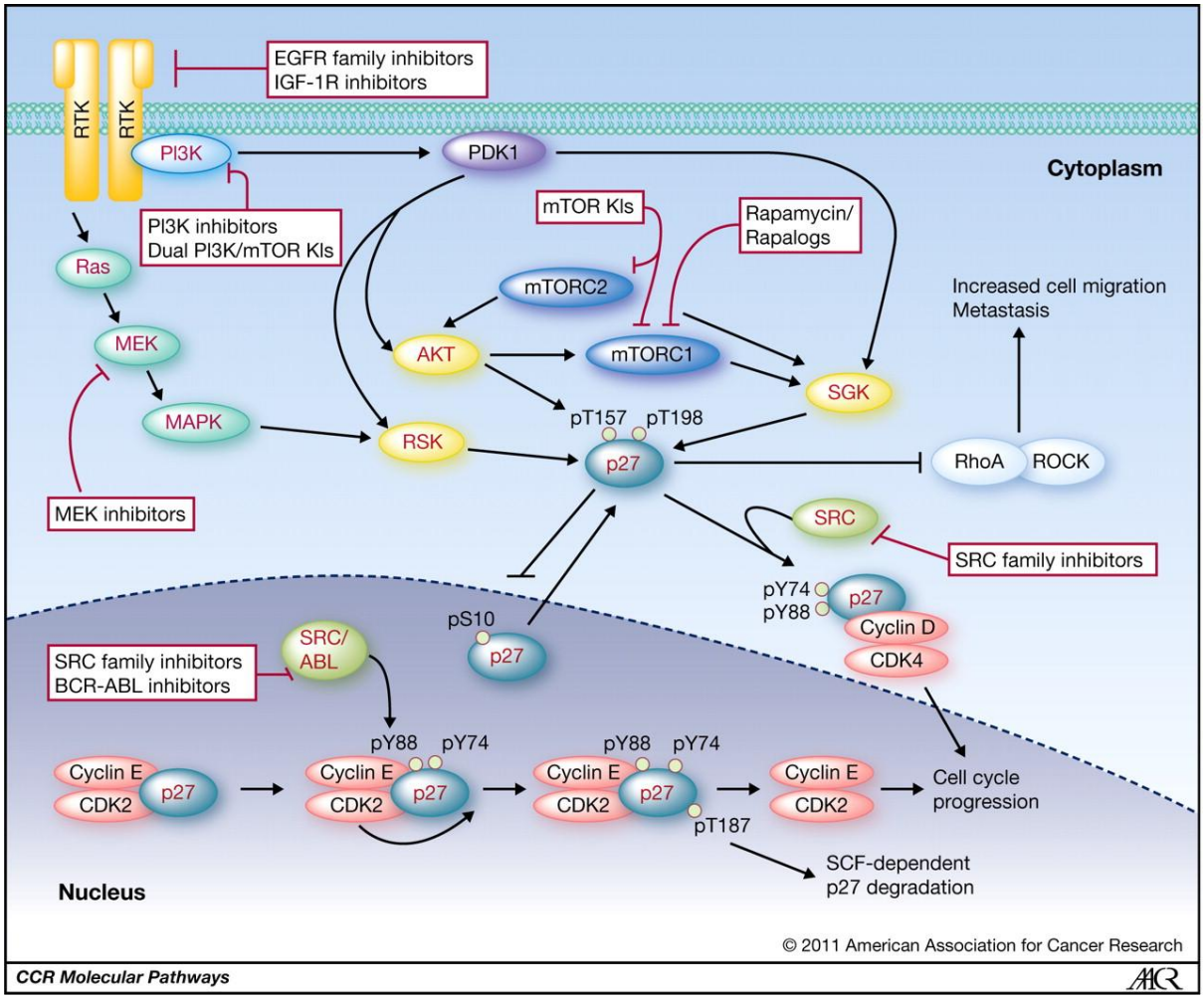


Figure 2.16: Multifaceted roles of p27 in the regulation of cell proliferation and cell migration

This schematic depicts p27 regulation and function as it relates to the protein's subcellular localization. While in the nucleus, p27 binds to and inhibits cyclin E-CDK2, preventing G1-S transit. Following SRC/ABL-mediated tyrosine phosphorylation and cyclin E-CDK2–mediated T187 phosphorylation, nuclear p27 is targeted for SCFSKP2-dependent degradation, allowing cell cycle progression. S10 phosphorylation promotes nuclear export; whereas in the cytoplasm, p27 may play several roles. Oncogenic signal transduction via PI3K/PDK1 or Ras/MAPK results in the hyperactivation of several AGC family kinases (including AKT, SGK, and RSK), which all mediate C-terminal phosphorylation of p27 at T157 and T198. These phosphorylation events cooperate to sequester and stabilize p27 within the cytoplasm, where it promotes cell proliferation (via cyclin D-CDK4 assembly) and cell motility (via inhibition of RhoA/ROCK signaling). Potential sites of targeted therapeutic intervention are highlighted in the figure (Wander et al., 2010).

p27 localization seems to be finely tuned during G1 progression. In early G1, as p27 translation decreases, nuclear export of p27 may be needed not only to relieve CDK2 inhibition, but also to promote p27-D type-cyclin-CDK assembly. Transient cytoplasmic retention of newly synthesized p27 is also facilitated in early G1 by PI3K effectors that phosphorylate p27 on T157 and/or T198 to both impair p27 import and promote cyclin D1-CDK4-p27 complex assembly (Wander *et al*., 2010).

Given the central roles of p27 in cellular proliferation and migration, it is no surprise that reduced or mislocalized p27, documented by immunohistochemical (IHC) analysis in primary tumors, is associated with poor clinical outcome in a diverse variety of human malignancies. Progressive p27 loss has been observed during the histopathologic progression of neoplasia from benign, to in situ, and invasive cancers of the breast (Catzavelos*et al*., 1997). One study showed that, relative to normal breast duct epithelia in which 95% of cells show high nuclear p27, premalignant atypical ductal hyperplasia shows modest p27 loss (85%), whereas ductal carcinoma in situ (40%) and invasive cancer (34%) show greater p27 loss (53). In ovarian cancer, lesions of low malignant potential showed intermediate p27 levels compared with high levels in normal epithelia, whereas highly aggressive cancers showed very low nuclear p27 (Sui *et al*., 2001; Wander *et al*., 2010).

Immunohistochemical analysis of p27 for prognostication of human cancers have been advocated. Reduced nuclear p27 is associated with adverse patient outcome in malignancies of epithelial, hematopoietic, and mesenchymal origin. The application of p27 as a clinical prognostic tool has been encumbered by the lack of uniformity in IHC staining protocols. Most IHC studies of p27 have evaluated only nuclear p27, and currently no accepted guideline exists for scoring cutoff values for “low” versus “high” (Wander *et al*., 2010).

2.5.12 p53 Protein

The TP53 gene, which encodes p53, is one of the most frequently mutated genes in human cancers. It is reported that approximately half of all cancers have inactivated p53 (Soussi and Beroud, 2001). The p53 protein has broad range of biological functions, including regulation of the cell cycle, apoptosis, senescence, DNA metabolism, angiogenesis, cellular differentiation, and the immune response. Suzuki and Matsubara (2011) reported various functions of p53 including transcriptional, posttranscriptional, and posttranslational roles.

Studies have shown p53 to be a transcription factor that targets many genes and microRNAs in response to cellar stress. The key role of p53 as a tumor suppressor is to block cell cycle progression and/or to induce apoptosis, in response to cellular stresses such as DNA damage. Impaired p53 activity promotes the accumulation of DNA damage in cells, which leads to a cancer phenotype. As a transcription factor, p53 forms a diverse and complex gene regulatory network (Suzuki and Matsubara, 2011).

More than 26,000 somatic mutation data of p53 appear in the international agency for research on cancer (IARC) TP53 database version R14 (http://www-p53.iarc.fr/) (Petitjean et al., 2007). Most TP53 mutations in human cancers result in mutations within the DNA-binding domain, thus preventing p53 from transcribing its target genes. However, mutant p53 has not only led to a loss of normal function of the wild-type protein but also led to new abilities to promote cancer (Suzuki and Matsubara, 2011).

Genetic instability is one of the most prominent features of malignant tumors. There are very sophisticated systems for detecting DNA damage and repairing the genome. p53 plays an important role in such “caretaker” systems. When p53 responds to DNA damage, it elicits either cell cycle arrest or apoptosis (Oren and Rotter, 2010).

It was shown in 1991 that induction of wild-type p53 can induce apoptosis in leukemia cells. Mice that have a specific p53 mutant lack the ability to induce cell cycle arrest, but retain the ability to induce apoptosis, allowing them to efficiently suppress oncogene-induced tumors, thus suggesting that the proapoptotic function of p53 may play a more important role in its antitumor effects than in its induction of cell cycle arrest as in Figure 2.17(Toledo *et al*., 2006).

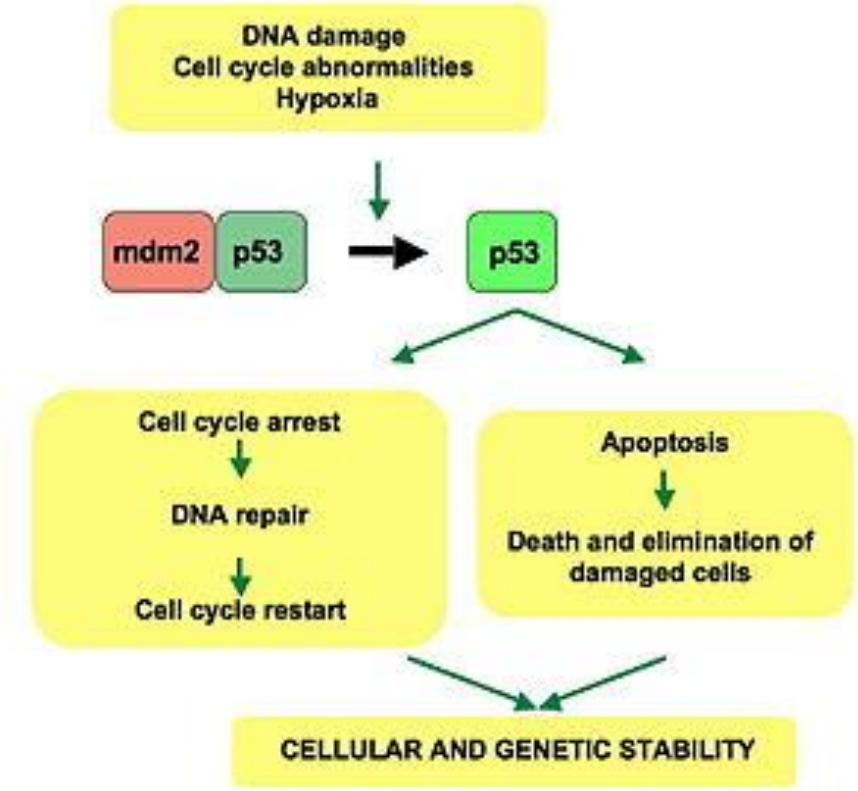


Figure 2.17: p53 Pathway

In a normal cell, p53 is inactivated by its negative regulator, mdm2. Upon DNA damage or other stresses, various pathways will lead to the dissociation of the p53 and mdm2 complex. Once activated, p53 will induce a cell cycle arrest to allow either repair and survival of the cell or apoptosis to discard the damaged cell.

2.5.13 Biomarkers in low resource setting

In the context of resource-constrained settings, the failure to establish and sustain cytology-based screening has necessitated research on operationally simple and less resource-intensive approaches for cancer prevention and control. Visual methods such as visual inspection with acetic acid and visual inspection with Lugol's Iodine provide immediate in vivo detection of visually apparent precancerous cervical lesions and the potential to link screening results and same-visit treatment by cryotherapy (or appropriate referral for cryotherapy-ineligible lesions). While visual inspection with acetic acid/visual inspection with Lugol's Iodine have been extensively evaluated and have high operational feasibility in the hands of nonphysician health providers, they miss anywhere between 20 and 50% of true disease due to variations in definitions of disease positivity, inherent subjectivity in test results, and challenges in quality assurance and control (Saharabudde*et al*., 2011).

There is a huge need for utilizing novel biologically-based approaches in resource-constrained settings of the developing world for improving access and accuracy of screening. CareHPV™ is a new assay developed by Qiagen that is a low-cost adaptation of the Digene hc2 assay and can be performed rapidly (<2 h) without access to running water or electricity, an ideal solution for operation in field settings. This assay has been shown to have performance characteristics approaching those of hc2, and in conjunction with simpler alternatives like visual inspection it may permit effective single visit strategies ('screen-and-

treat') by same-day results and linkage to cryotherapy. Yet, further research on adaptation of these strategies is needed to avoid overtreatment, given the different age distributions of HPV prevalence worldwide.

Novel biomarkers that reflect measurement of an advanced disease process end point, such as overexpression of p16ink4a or HPV E6 protein detection, are also being evaluated in these settings, with the goal of achieving an optimal balance of sensitivity and specificity for very infrequent testing. Additional efforts are also being undertaken to evaluate biomarker assays using noninvasive and user-operated screening methods (e.g., self-sampling or urine-based sampling) that can address challenges in improving access to cervical cancer prevention services in these settings (Saharabudde*et al*., 2011).

2.6 Natural History of Cervical Cancer

The fact that a high number of women are infected with high-risk HPV during lifetime but only a small proportion of them develop cervical cancer indicates that cervical cancer is a rare complication of an infection with HPV (Fernandes*et al*., 2013).

Approximately in 20% of women infected with high-risk HPV a CIN lesion will develop within 2-4 years after acquisition of the virus. This may lead to maintenance of CIN or progression from CIN 1 to CIN 3. Invasive cervical cancer ultimately manifests in a small subset of cases. Most of the premalignant lesions, especially CIN 1 and CIN 2 will regress spontaneously. Regression rates varying from 30% to 62% in women with CIN 1 and 17% to 54% in CIN 2 are reported (Fernandes*et al*., 2013). Several studies have reported that women with high-risk HPV positive premalignant lesions were more likely to progress than women with high-risk HPV negative lesions and therefore, progression of CIN lesions is assumed to be associated with a persistent high~risk HPV infection (Carr and Sellors, 2004; Saharabudde*et al*., 2011; Fernandez *et al*., 2013; Nayar*et al*., 2014) as shown in Figure 2.14

and Figure 2.15. This was also shown in case-control studies in which HPV DNA could be detected persistently in cytological normal archival smears of women years before cervical cancer subsequently developed (Ibeanu, 2011).

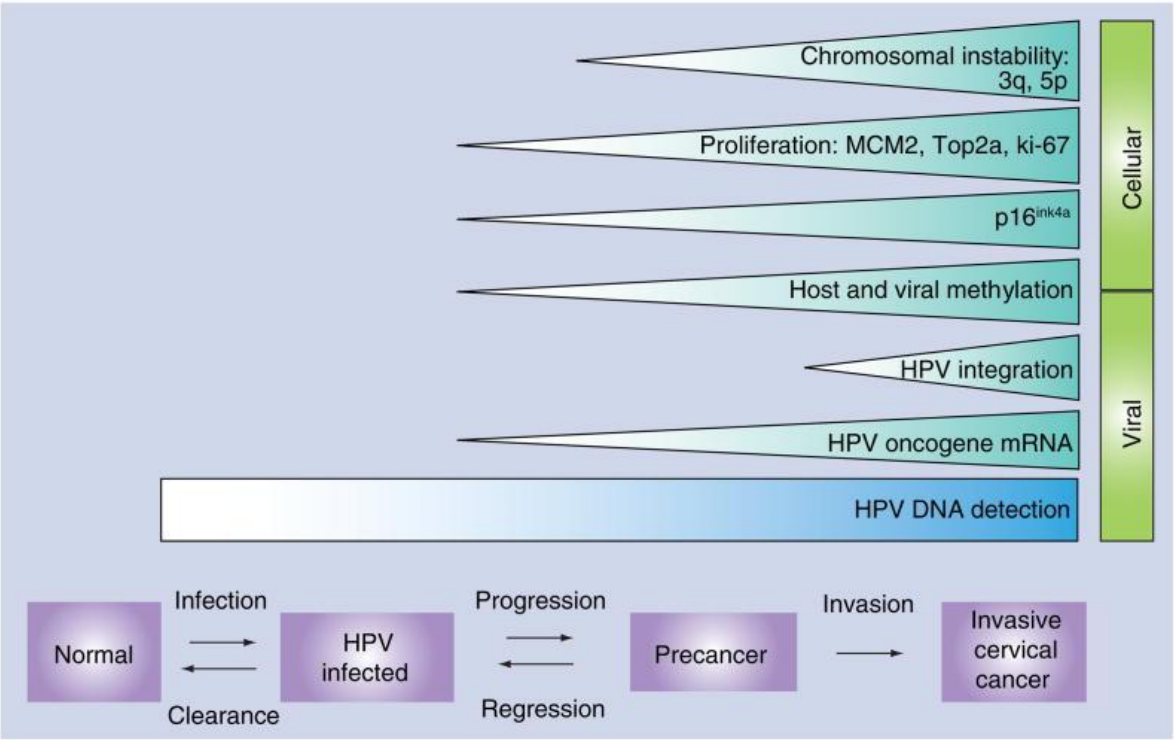


Figure 2.18: Human papillomavirus natural history and cellular and viral biomarkers used in cervical cancer screening (Saharabudde*et al.*, 2011)

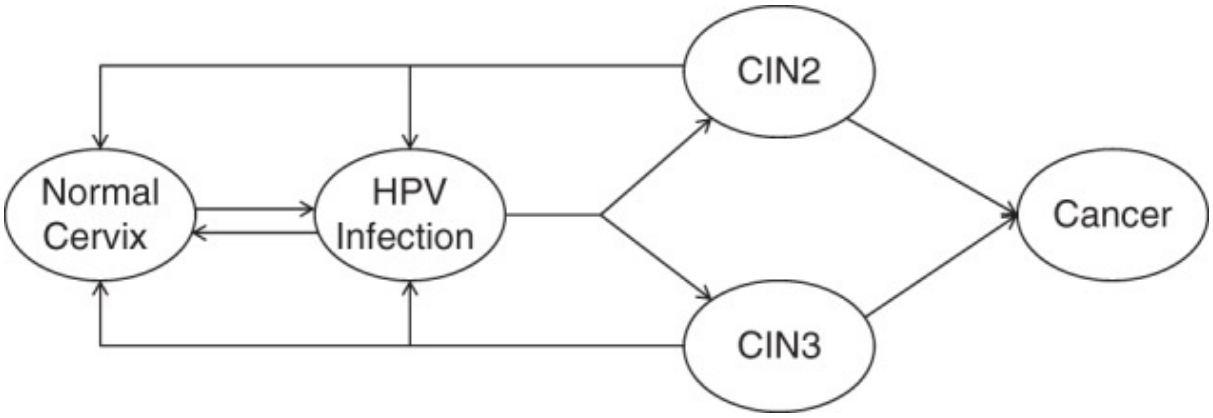


Figure 2.19: Schematic progression of Human Papillomavirus (HPV) infection to Cervical Cancer (Campos *et al.,* 2014)

The major problem in the development of cervical cancer through the different premalignant stages is the difficulty in predicting which lesion will progress or regress. The clinical usable definition of a persistent infection has not yet been defined and the value of methods of detecting lesions with a high likelihood of progression is not yet available. It has been proposed that high-risk HPV clearance results in regression of CIN, but this process has never been studied properly. Moreover, it is necessary to know if clearance depends on the severity of the underlying lesions (Nayar*et al.*, 2014).

The interval between the first manifestation of CIN 1 and the development of cervical cancer is estimated to be about 13 years. This long period indicates that, in addition to an oncogenic HPV infection and most probably immunological factors, changes in the cellular genome are mandatory. This theory is substantiated by the fact that the viral oncogene products E6 and E7 bind the gene products of the human tumour suppressor genes p53 and Rb (Ibeanu, 2011; Nayar*et al.*, 2014). This interaction in proliferating cells leads to dysregulation of cell cycle control and shortcomings in DNA repair, which might result in genetic instability and an increased risk of obtaining changes and mutations (activation of oncogenes, loss of tumor suppressor genes, activation of telomerase) essential for progression to a malignant phenotype as shown in Figure 2.16. Thus, HPV is capable of initiating cancer through the disruption of multiple tumour-suppressor pathways but alone is not sufficient for the development of the fully transformed cancer phenotype (Carr and Sellors, 2004; Fernandez *et al.*, 2013; Litwin*et al.*, 2017).

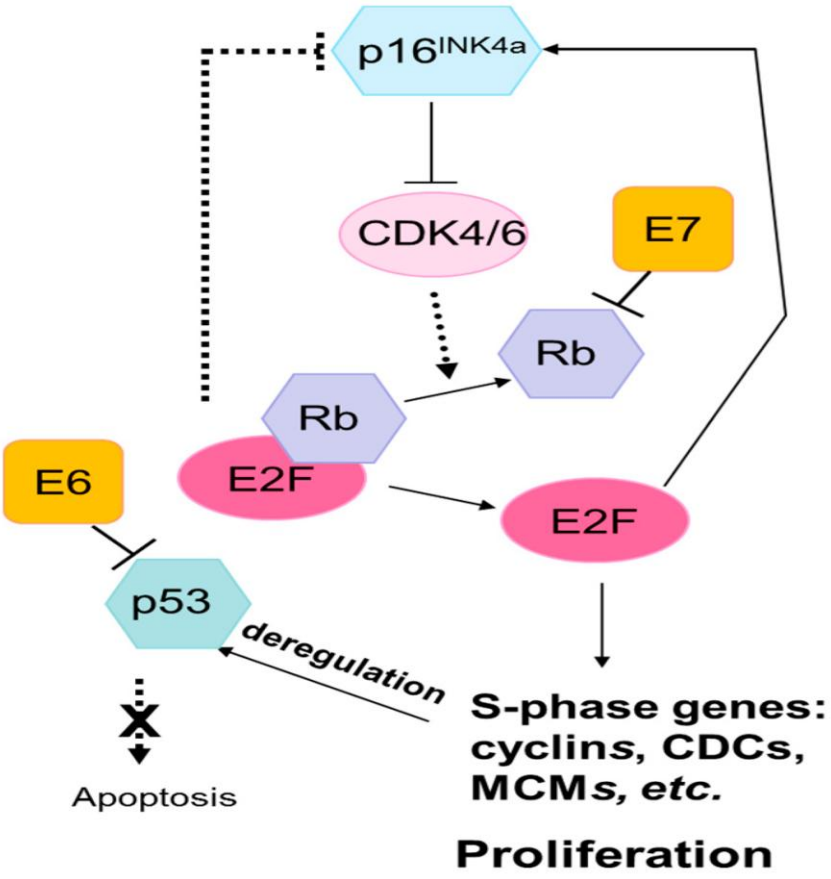


Figure 2.20: The Rb and p53 pathways in HPV Infection

These pathways are disrupted by the human papilloma virus (HPV) oncoproteins E7 and E6, respectively. The HPV E7 protein binds to Rb with high affinity, disrupting its interaction with the transcription factor E2F. This results in the release and activation of E2F, driving expression of S-phase genes and cell cycle progression. P16INK4A is a cyclin-dependent kinase inhibitor that regulates the cell cycle by inactivating cyclin-dependent kinases involved in Rb phosphorylation. Upregulation of p16INK4A is induced by HPV-mediated disruption of E7, leading to the accumulation of p16INK4A in HPV-transformed cells. The HPV E6 protein inhibits apoptosis by targeting the tumor suppressor protein, p53, for degradation. HPV E6 inhibition of p53 promotes cell proliferation and can lead to genomic instability and the accumulation of somatic mutations. Abbreviations: Rb, retinoblastoma protein; p16INK4A, cyclin-dependent kinase inhibitor 2A; CDK, cyclin-dependent kinases;

E2F, E2F transcription factor; CDC, cell-division-cycle genes; MCM, minichromosome maintenance family (Litwin, *et al*., 2017)

2.7 Prevention and Control of Cervical Cancer

Different methods of cervical cancer prevention of control have been developed andimplemented worldwide. These methods include early diagnosis and treatment ofprecancerous lesions and have led to a significant reduction in the burden of the disease. Screeningfor precancerous lesions can be done in several ways including, cervical cytology (Papsmear), and visual inspection of the cervix with acetic acid [VIA] or testing for HPV DNA.Each of these methods has specific advantages, disadvantages and health systemsrequirements that countries should consider when planning screening programmes.Vaccinating girls and women before sexual debut, and therefore before exposure to HPVinfection, provides an excellent opportunity to decrease the incidence of cervical cancer overtime. Increasing awareness of women about risk of cervical cancer and of benefits ofscreening programme is crucial in prevention of the disease.

2.7.1 Screening: definition and principles

Screening is defined as a procedure used to identify specified diseases or particularcondition among asymptomatic individuals. In contrast, diagnostics is defined as applicationof variety of tests to symptomatic individuals who actively seek health-care services toidentify the cause of their symptoms. The distinction between screening and a diagnostictest is whether the test is offered without individual consideration (Vesco*et al*., 2011). Screening tests

areapplied to large populations, therefore they should be relatively inexpensive, convenient,painless and safe (Saket*et al*., 1991). For this reason they often have higher margins of error and are lessaccurate than diagnostic tests.

The sensitivity of screening test is a measure of how good the test is at identifyingindividuals with a given disease. Sensitivity is defined as proportion of persons with a givendisease who are screened as positive (Saket*et al*., 1991). The screening result is true positive when thescreening result is positive and the person has the disease. The screening result is falsepositive when the screening result is positive but the person does not have the disease.

The diagnostic tests should have very high sensitivity to detect the disease, whereassensitivity and specificity are balanced between each other to detect people with and withoutlikelihood of disease and affordable costs.The specificity of the screening test is a measure of how good the test is at identifyingunaffected subjects. Specificity is defined as the proportion of individuals without thedisease who get a negative screening test result (Saket*et al*., 1991; Vesco*et al*., 2011). The screening result is true negative whenthe screening test is negative and the person does not have the disease and, the screening testis false negative when the screening test is negative but the person has the condition.

Sensitivity and specificity are inversely related to each other. When sensitivity is increased,more persons with the disease are detected but also more persons who do not have thedisease receive a positive screening result which is considered as false positive.Positive and negative predictive values depend not only on the sensitivity and specificity ofscreening test but also on the prevalence of screened condition. The positive predictive valueis probability of the disease in the subjects with positive result. The positive predictive valueis higher for common disease than for rarer diseases. The significance of a positivepredictive value depends very much on the consequence of a positive test. If it simply isfollowed by

repetition of the screening test, the low positive predictive value might be wellacceptable. If it is followed by a potentially harmful diagnostic examination it is importantto achieve a high predictive value (Saket*et al*., 1991).

To be considered effective, a screening test mustsatisfy the requirements of efficacy and effectiveness of early detection. Efficacy ofscreening means that the test must be able to detect the target condition earlier than it wouldbe without screening and with sufficient accuracy to avoid producing large number of false– positive and false –negative results. Effectiveness of early detection means that personswith disease who are detected early should have a better clinical outcome than those who aredetected without screening (Bell *et al*., 1995). In offering screening service for large number of symptom-free persons, and before starting the screening programme, very firm evidence is required toconfirm that early diagnosis and any subsequent treatment will be better without harm. Thepossible harms can be in form of false positive screening test result, a wrong diagnosis,treatment which may do more harm than good, labeling people and false-negative findingsthat give false assurance (Lefebvre*et al*., 1988).False positive findings cause anxiety for healthy people, resultin exposure of screened people to further examination, which may have risks.

Screening test has some psychological and social harms on screened women; these involveanticipated discomfort or perception of adverse effects of screening test; unpleasantinteractions with health care workers, anxiety over the results of a screening testimplications of a positive screening test, and consequences of being labeled as sick or atrisk of cervical cancer (Bell *et al*., 1995). The result of screening test has different impacts on the screenedpeople. People with a true positive finding and for whom the death is postponed, largelybenefit from screening, but the benefit for those who have true positive finding but forwhom death is not postponed, the value of screening is disputable. A false

positive resultcauses moderate adverse effect, while false negative result causes minute undesirable effectand the true negative result has questionable value (Vesco*et al.*, 2011).

The objective of screeningprogrammes is to reduce morbidity and mortality, and to improve the quality of life in thepopulation (Vesco*et al.*, 2011).

The criteria of screening programme, conditions to be screened and screening test werethoroughly described by Andermann,*et al*.(2008) as follows:

A. The Wilson- Jungner criteria for appraising the validity of a screening programme

1. The condition being screened for should be an important health problem.
2. The natural history of the condition should be well understood.
3. There should be a detectable early stage.
4. Treatment at an early stage should be of more benefit than at a later stage.
5. A suitable test should be devised for the early stage.
6. The test should be acceptable.
7. Intervals for repeating the test should be determined.
8. Adequate health service provision should be made for the extra clinical workload resulting from screening.
9. The risks, both physical and psychological, should be less than the benefits.
10. The costs should be balanced against the benefits.

B. The criteria for condition to be screened

1. The condition should be an important health problem.

2. The epidemiology and natural history of the condition, including development fromatent to declared disease, should be adequately understood and there should be adetectable risk factor, disease marker, latent period or early symptomatic stage.
3. All the cost-effective primary prevention interventions should have beenimplemented as far as practicable.
4. If the carriers of a mutation are identified as a result of screening the natural historyof people with this status should be understood, including the psychologicalimplications.

C. Criteria of screening test

1. There should be a simple, safe, precise and validated screening test.
2. The distribution of test values in the target population should be known and a suitablecut-off level defined and agreed.
3. The test should be acceptable to the population.
4. There should be an agreed policy on the further diagnostic investigation ofindividuals with a positive test result and on the choices available to thoseindividuals.
5. If the test is for mutations the criteria used to select the subset of mutations to be coveredby screening, if all possible mutations are not being tested for, should be clearly set out.

D. The treatment for screened condition

1. There should be an effective treatment or intervention for patients identified throughearly detection, with evidence of early treatment leading to better outcomes than latetreatment.
2. There should be agreed evidence-based policies covering which individuals should beoffered treatment and the appropriate treatment to be offered.

3. Clinical management of the condition and patient outcomes should be optimized inall healthcare providers prior to participation in a screening programme.

2.7.2 Criteria of screening programme

UK National Screening Committee published the following criteria for screeningprogramme (National Screening Committee, 2016):

1. There should be evidence from high-quality randomized controlled trials that thescreening programme is effective in reducing mortality or morbidity. Wherescreening is aimed solely at providing information to allow the person being screenedto make an informed choice, there must be evidence from high-quality trials that thetest accurately measures risk. The information that is provided about the test and itsoutcome must be of value and readily understood by the individual being screened.
2. There should be evidence that the complete screening programme is clinically,socially and ethically acceptable to health professionals and the public.
3. The benefit from the screening programme should outweigh the physical andpsychological harm (caused by the test, diagnostic procedures and treatment).
4. The opportunity cost of the screening programme (including testing, diagnosis andtreatment, administration, training and quality assurance) should be economicallybalanced in relation to expenditure on medical care as a whole (i.e. value for money).
5. There should be a plan for managing and monitoring the screening programme and anagreed set of quality assurance standards.
6. Adequate staffing and facilities for testing, diagnosis, treatment, and programmemanagement should be available prior to the commencement of the screeningprogramme.

7. All other options for managing the condition should have been considered (forexample, improving treatment and providing other services), to ensure that no morecost-effective intervention could be introduced or current interventions increasedwithin the resources available.
8. Evidence-based information, explaining the consequences of testing, investigation,and treatment, should be made available to potential participants to assist them inmaking an informed choice.
9. Public pressure for widening the eligibility criteria for reducing the screeninginterval, and for increasing the sensitivity of the testing process, should beanticipated. Decisions about these parameters should be scientifically justifiable tothe public.
10. If screening is for a mutation, the programme should be acceptable to peopleidentified as carriers and to other family members.

2.8 Screening: Visual Approaches

Given the challenges of implementing high-quality cytology-based services, especially in developing countries, there is significant interest innew approaches to screening for precursor lesions. Two approaches of particular interest are visual screening and HPV testing.

2.8.1 Visual Inspection with Acetic Acid (VIA)

This screening approach involves swabbing the cervix with an acetic acid (vinegar) solution prior to visual examination. Differences in precancerous cell structure and absorption rates make abnormal cells temporarily turn white when exposed to this solution. Alternatively, some visual screening approaches have used the application of an iodine-based solution (Lugol's solution) as a means of staining normal cervical cells brown, leaving the abnormal cells with a yellow or unstained appearance. Many aspects of VIA make it a promising approach for use in low resource settings. For example, costs associated with launching and

sustaining VIA screening are lower than those associated with other screening methods. VIA also is a relatively simple, low-tech approach that is minimally reliant upon infrastructure for its adequate performance, assuming that treatment services are in place. Non-physicians can perform the procedure, provided that they receive adequate and ongoing training. Furthermore, results of the procedure are available immediately, making it possible, in principle, to provide treatment during the same visit.

Several studies examining the accuracy of VIA have found that the technique has the potential to be reasonably accurate. Differences in study protocols, populations studied, and outcomes, make it difficult to summarize results, however. In addition, many studies are subject to verification bias because the reference test was not performed on all study subjects, including women with negative screening test results. This bias tends to inflate the estimated sensitivity of a screening test. Nevertheless, some broad conclusions regarding VIA's utility in low-resource settings can be made based on results of several studies. In general, the sensitivity of VIA in detecting high-grade dysplasia is at least equal to that of cytology, while VIA's specificity is somewhat lower (Ahmed, 2010).

Lesions viewed by VIA vary in size, thickness, opacity, and border definition (larger, thicker, more opaque lesions with clear border suggest more severe disease). To implement appropriate medical protocols, health care providers must carefully consider the features of a lesion. Clearly, a significant challenge to effective implementation and standardization of VIA is training health providers to recognize the often-subtle characteristics that differentiate degrees of dysplasia. The feasibility of utilizing VIA for wide-scale screening is uncertain, and to a large extent will be determined by the effectiveness of training and monitoring efforts.

The specificity of VIA remains a cause of concern due to the potential forover treatment of women with false-positive test results. Over treatment may result in additional health risks to women, as well as overburdening the health care system and increasing costs. VIA also is less effective for screening women in their fifties because of the tendency for the squamocolumnar junction (the point at which columnar cells meet ectocervical squamous cells of the cervix) to recede into the cervical os, making observation of lesions difficult. (Pap smears also are more difficult to obtain in post-menopausal women.) Despite these drawbacks, VIA shows promise as an option for identifying precancerous lesions in many settings, either in conjunction with or as an alternative to other screening approaches.

2.8.2 Visual Inspection with Magnification

VIA with magnification (VIAM) uses the AviScope to examine the cervix after application of acetic acid. An evaluation of an earlier version of the device indicated that VIAM may achieve sensitivity and specificity of over 90 percent in identifying pre-invasive cervical lesions. Preliminary findings from an ongoing study in Calcutta, India, indicate that VIAM has a sensitivity of 69 percent and specificity of 82 percent. It is not yet known whether use of the AviScope offers a significant advantage over VIA, although the potential for increased specificity is of particular interest (Ahmed, 2010).

2.8.3 Other Approaches to Visual Inspection

One other approach to visual inspection also is noteworthy. Cervicography involves photographing the cervix after application of an acetic acid wash. The developed photographs, called cervigrams, are projected as slides and interpreted by specially trained colposcopists. Like other visual inspection methods, the sensitivity of Cervicography is comparable to cytology, while its specificity appears to be lower. This approach offers the advantage of providing a permanent record of the cervix. Cervigrams can also be helpful as

educational tools. Cervicography, however, is relatively expensive and requires a reliable logistics infrastructure.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Background of the Study Area

The Ahmadu Bello University Teaching Hospital (ABUTH) Zaria is a 700-bed hospital engaged inclinical service, training and referralof cases for specialized clinical services. The hospitalis located in the northwest geopolitical zone of Nigeria and it offers medical services to the population of Kaduna state and neighbouring states of Zamfara, Jigawa, Katsina and Niger. It is also a referral center for radiotherapy and oncology services in the Northern part of the country and thus many patients are referred to the hospital for specialist cancer services. The Pathology Department of the Ahmadu Bello University Teaching Hospital Zaria is the oldest in Northern Nigeria and receives specimens from across Kaduna and contiguous states as well as referral from other Pathology Departments in the country. This

study was carried out in the Family planning and Gynaecologyclinics, the Pathology Department of theABUTH Zaria and the DNA Labs Kaduna.

3.2 Study Design

The study was a cross-sectional type where prevalence of abnormal smears, HPV DNA and possible associated risk factors were assessed.

3.3 Study Population

The study population comprised of women between the ages of 18 – 59 years attending Family Planning and Gynaecologyclinics of ABUTH Zaria, Nigeria.

3.4 Sample Size Determination

Sampling size for the study wascalculated based on the formula below (Kish, 1965):

$$\text{Sample Size } n = \frac{Z^2 PQ}{d^2}$$

Where:

n= Sample size

z= degree of confidence

P = Prevalence. From a previous study by Ahmed *et al*. (2013), prevalence of squamous intraepithelial lesions of 15.7% in Jalingo was reported and was used to calculate the sample size.

Q = Complementary prevalence (1 – P)

d = Precision

z= (1-α/2) = 1.96

P= 0.157

Q =0.843

d= 0.05

$$n = \frac{(1.96)^2 x 0.157 x 0.843}{(0.05)^2}$$

n= 203

Therefore, deliberate over-sampling was done and sample size of 500 was used.

3.5 Sampling Method

This study adopted the purposive sampling method. All consecutive consenting participants wererecruited until the sample size was achieved.

3.6 Inclusion Criteria

a) Women between the ages of 18 and 59 years attending Family Planning and GynaecologyClinics of ABUTH Zaria, Nigeria.

b) Participants must be sexually active.

3.7 Exclusion Criteria

a) Women with history of cervical cancer;

b) Pregnant women;

c) Women with an obvious lesion on the cervix.

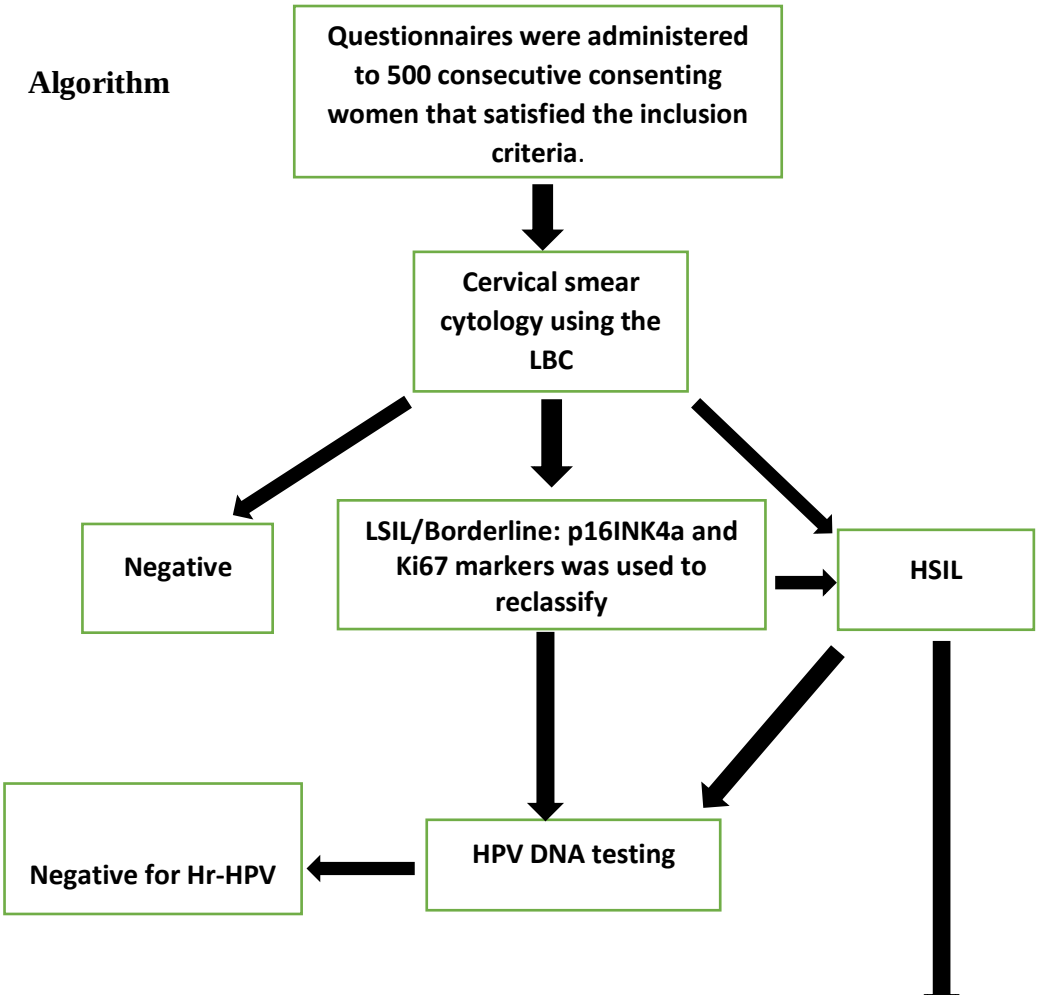
3.8 Materials

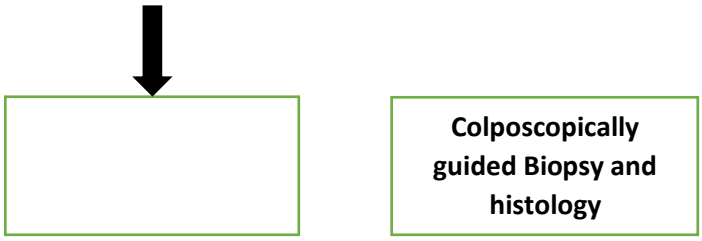
1. Cervex® brushes (Rovers Medical Devices B.V 5347 KV Oss, The Netherlands
2. LiquiPrep® LBC Kits (P 121514V, LGM Inc, Melbourne FL, U.S.A)
3. CytospinUniversal 32(Hettich, LD007-ZR-001, Germany)
4. Vortex (LGM®, XHB Votex, California, USA)
5. Micropipettes (Labline® Eco, Mombai, India)

6. Immunohistochemistry chamber (Binder®, KBF 115, USA)
7. DAB Kit (DBDE TKIT0418149H, DBBiotech, Slovakia)
8. Ki-67 (Lot No. A2180, Biorbyt®, United Kingdom; Dilution 1:100)
9. p16INK4a (DB062-01-06A, DBBiotech® Slovakia; Dilution 1:100)
10. Permanent Red + Substrate (Lot No. 0000017848, Cellmarque®, U.S.A.)
11. AccuPrep® Genomic DNA Extraction Kit (Cat No. K-3032, Bioneer Corporation, South Korea)
12. Primers: MY09/MY11 and GP5+/6+ primers
13. Accu-Power HotStart Premix (P/No. 292883, Bioneer Corporation, South Korea)
14. Thermal cycler (T100® Thermal cycler, Bio Rad, U.S.A.)
15. Sequencing machine (Beckman Coultier CEQ 2000XL, Fullerton, CA, U.S.A.)

3.9 Protocols

3.9.1 Algorithm





3.9.2 Specimen collection and storage

Rovers® Cervex brush cell sampling device (RoversMedical Devices B.V 5347 KV Oss, The Netherlands)was used for specimen collection. Specimens were obtainedby inserting the Cervex brush into the cervical canaland rotating it four times in a clockwise direction to collectall the cervical epithelial cells which adhered to theflat sides of the bristles.The head of the brush was then inserted into the collection vial containingpreservative fluid. Liquid-based cytology system (Liqui-PREP by LGM International, Inc, Melbourne, FL, USA)for collection and transport of cervical specimen wasused.

Each specimenwas mixed using Vortex and then each specimen poured into Liqui-Prep® Cleaning solution. Thereafter the mixture was centrifuged for 10 minutes to remove mucous and other non-diagnostic materials and cellular pellet was produced and Liqui-Prep® cellular

base is added and pipetted onto a glass slide.Staining was done using the Papanicolaou technique and mounted with DPX® and examined using the light microscope.

3.9.3 Papanicolaoustaining method

Samples obtained were stained according to the method of Stevens *et al*. (2002) using Papanicolaou staining technique (Appendix I).

The smears wereremoved from the fixative and rinsed in descending grades of alcohol (80%, 70% and 50%) and in water for 10seconds each. After this dehydration, staining each slide with Harris Haematoxylin for 2 minutes was done. Then the slide was then rinsed in water for 1-2 minutes. Subsequently it was differentiated in 1% acid alcohol for a few seconds until only the nuclei retained the stain. Then slides were washed and blued in tap water for 3 seconds each and transferred to 70% alcohol for a few seconds. Then transferred to 95% alcohol for a few seconds and subsequently stained in Orange Green 6 for 2 minutes. This is then rinsed in two changes of 95% alcohol and stained in Eosin Azure 50(EA 50) for 2 minutes and rinsed in two changes of 95% alcohol. Slides were then dehydrated in absolute alcohol, cleared in two changes of xylene for two minutes and mounted using DPX and covered with cover slips (24 x 50 mm cover slips.).

3.9.4 TheBethesdaSystemofclassification

The smears were examined using a light microscopeand reported using the Bethesda 2014Classification (Appendix II):

INTERPRETATION/RESULT

Negative For Intraepithelial Lesion or Malignancy,

(when there is no cellular evidence of neoplasia, state this in the General Categorization above and/or in the Interpretation/Result section of the report, whether or not there are organisms or other non-neoplastic findings).

- Organisms:

* *Trichomonas vaginalis*

* Fungal organisms morphologically consistent with *Candida spp*

* Shift in flora suggestive of bacterial vaginosis.

* Bacteria morphologically consistent with *Actinomyces spp.*

* Cellular changes consistent with *Herpes simplex* virus

- Other Non Neoplastic Findings(Optional to report; list not inclusive):

* Reactive cellular changes associated with

  - inflammation (includes typical repair)

  - radiation

  - intrauterine contraceptive device (IUD)

* Glandular cells status post hysterectomy

* Atrophy

Other

- Endometrial cells (in a woman >= 40 years of age)

(Specify if 'negative for squamous intraepithelial lesion')

Epithelial Cell Abnormalities

- Squamous Cells

* Atypical squamous cells

  - of undetermined significance (ASC-US)

  - cannot exclude HSIL (ASC-H)

* Low grade squamous intraepithelial lesion (LSIL)

(encompassing: HPV/mild dysplasia/CIN 1)

* High grade squamous intraepithelial lesion (HSIL)

(encompassing: moderate and severe dysplasia, CIS, CIN 2

and CIN 3)

- with features suspicious for invasion (if invasion

is suspected)

* Squamous cell carcinoma

- Glandular Cells

* Atypical

- endocervical cells (not otherwise specified (NOS) or specify in comments),

- endometrial cells (NOS or specify in comments),

- glandular cells (NOS or specify in comments)

* Atypical

- endocervical cells, favor neoplastic

- glandular cells, favor neoplastic

* Endocervical adenocarcinoma in situ

* Adenocarcinoma:

- endocervical

- endometrial

- extrauterine

- not otherwise specified (NOS)

Other Malignant Neoplasms: (Specify)

3.9.5 Histological technique

Gross and histological processing of surgical specimens were performed according to standardised surgical pathology protocols, histological diagnoses were made using the WHO classification. The histological techniques (Bancroft and Stevens, 1999) as shown in Appendix III involveTissue fixation in 10% neutral buffered formalin and then gross examination and tissue sectioning done by the pathologist.

Thereafter tissue dehydration using graded ethanol concentration (70%, 95%, 100%) was done, tissue clearing with xylene and paraffin wax embedding carried out in a mould. Microtomytrimming and sectioning at 5µ with a rotary microscope was done. Sections were placed in floating out tissues in water bath and dried on hot plate.

3.9.6 Haematoxylin and eosin staining protocol

Dewaxing of sections, rehydration through graded concentrations of ethanol (100%, 95%, 70%) was done and then stained in Harris Haematoxylin for 5 – 15mins. Slides were washed well in running tap water until they turn blue for five minutes or less. Differentiate in 1% acid alcohol for 5 – 10 secs and wash well in tap water until sections are again blue for 5mins or less. Stain in Eosin for 10mins and wash in running tap water for 1 – 5mins. Thereafter dehydrate through alcohols, clear and mount.

3.9.7 Immunocytochemistry

Immunocytochemical stains comprising Ki67 (Biorbyt®, UnitedKingdom) and p16INK4a (DBBiotech®, Slovakia) was done on liquid based cytology specimens using the protocoloutlined below as shown in Appendix IV.

Smear made on charged glass slide and blocking of endogenous enzymes done by applying 3% Hydrogen peroxide for 10 minutes.Wash in TrisBuffered Saline (TBS, pH 7.5). Treat

with epitope retrieval buffer (pH 6) at 95˚C for 20 minutes and wash in TBS.Incubate in anti-P16 (DBBiotech®, Slovakia) for 60 minutes and wash in TBS.

Incubated smear in Mouse + Rabbit HRP (DBBiotech®, Slovakia) for 30 minutes and wash in TBS. Treat smear with 3,3-Diaminobezidine (DAB) + Chromogen Substrate (DBBiotech®, Slovakia) for 7 minutes and wash in distilled water.

Incubate smear in alkaline phosphatase anti-Ki67 (Biorbyt®, United Kingdom) for 60 minutes and wash in TBS. Treat smear with Permanent Red + Substrate (Cellmarque®, U.S.A.) for 5 minutes wash in distilled water.Counterstain in MayersHaematoxylin for 2 minutes.The slide was air-dried and mounted with DPX and reported under the microscope.

All immunostained slides wereanalysed and reported and samples were considered positive when p16 andKi-67 immunoreactivities were revealed within the same cell (i.e., acytoplasmic brown staining for p16, together with a nuclear redstaining for Ki-67). The presence of at least one dual-stained cell(s)was used as a cutoff to rate the sample as positive test (Uijterwaal*et al*., 2014).

3.9.8 DNA extraction

DNA from exfoliated cervical cells collected in thepreservative fluid (Liqui-PREP) were extracted using AccuPrep® Genomic Extraction Kit (Bioneer, South Korea) according to manufacturer's instructions. (Appendix V).

20 µl of Proteinase K was added to a clean 1.5 ml tube and 200 µl of sample was added to the tube containing Proteinase K.200 µl of GB Buffer was added to the sample and mixed immediately by vortex mixer.

Incubation at 60 degrees Celsius for 10 min was carried out and 400 µl of absolute ethanol was added and mixed well by pipetting.After this step, sample was spun down to get the drops clinging under the lid.Carefully transferred the lysate into the upper reservoir of the Binding column tube (fit in a collection tube)without wetting the rim.The tube was closed and centrifuged at 8,000 rpm for 1 min.Binding column tube was closed to avoid aerosol formation during centrifugation. Where lysate hasnot completely passed through the column after centrifugation, we centrifuged again at a higher speed (>10,000rpm) until the binding column tube was empty.The solution was discarded from the collection tube into a disposal bottle and reuse the collection tube.500 µl of WA1 Buffer was added without wetting the rim, the tube closed, and centrifuged at 8,000 rpm for 1 min.

3.9.9 Detection and typing of HPV

All the cervical specimens were tested for the presence of oncogenic HPV DNA using a nested Polymerase Chain Reaction (nPCR) with GP5+/GP6+ (GP5+[5′ TTTGTTACTGTGGTAGATACTAC-3′] and GP6+ [5′-GAAAAATAAACTGTAAATCATATTC-3′]) and PGMY 09/11 consensus primers which amplifies a 150 bp fragment of the L1 HPV genomic region. Accu-Power HotStart Premix (Bioneer Corporation, South Korea) was used for the PCR. Genotypic identification was achieved by direct sequencing using the Gp 6+ oligoprimer. Thermal cycler (Bio Rad) and Sequencing machine (Beckman Coultier CEQ 2000XL) were used for PCR and sequencing respectively. Sequence alignments was performed against various standard HPV genotype sequences stored in the GenBank database by on-line BLAST analysis to arrive at specific genotyping.

3.10Data collection

Questionnaires were administered for data collection. Questions are both closed ended and open ended as required, providing information about basic demographic data and risk factors of cervical cancer were assessed (Appendix VI).

Questionnaires wereself-administeredfor literate participants; and interviewer-administered for those that were not literate. All participants wereassured of the confidentiality of their responses. The questionnaire was pretested and validated before applying at the study site.

3.11 Data Analysis

The data entry and analyses were done using statistical program for social sciences (SPSS)® version 23.0.Univariate analysis wasconducted to compute frequencies and proportions. Bivariate analysis using Chi square test at 95% confidence interval wasconducted to determine the association between HPV Infection and the risk factors for cervical cancer. A p-value of ≤ 0.05 wasconsidered statistically significant. Tables, proportions and percentages as well as charts were used to summarize data obtained from the study.

3.12Ethical Consideration

Ethical clearance was obtained from the Health Research Ethics Committee of the ABUTH, Zaria for the study (Appendix VII). Consent was also sought from the participants prior to enrollment into the study and confidentiality was maintained.

3.13Limitations

The study is hospital based and may not necessarily accurately represent the general population. Secondly, not all the participants with high grade lesions accepted biopsy and histology.

Other biomarkers like p21, p27 and p53 could have added value to this work but they were not considered at conception due to cost limitation. These may constitute areas of future studies which will further increase the understanding of the biology of cervical precancer and prevention.

CHAPTER FOUR

4.0 RESULTS

A total of five hundred women between the ages of 18 and 59 years were recruited for the study and the mean age was 40.5 ± 9.7 years. Most respondents were in the fourth and fifth decades, 33.2 and 30.4% respectively. Only 1.2% of respondents are less than 20 years of age (Table 4.1).

Table 4.1: Frequency distribution ofrespondents by age (n=500)

Age group (Yrs)	Frequency	Percent(%)
<20	6	1.2
20 - 29	65	13.0
30 - 39	166	33.2
40 - 49	152	30.4
50 - 59	111	22.2
Total	500	100.0

Table 4.2: Frequency Distribution of Respondents' Ethnic Group by Age

Age Group (Yrs)	Ethnic Group				Total
	Hausa	Igbo	Yoruba	Others	
<20	0	0	0	1	1
20-29	29	1	3	0	33
30-39	36	4	8	21	69

40-49	24	4	7	18	53
50-59	19	2	10	24	55
Total(%)	108(51.2)	11(5.2)	28(13.3)	64(30.3)	211

Most of the respondents were of Hausa ethnic group (51.2%), Yoruba and Igbo comprised 13.3% and 5.2% respectively. Other tribes like Kataf, Angas, Bachama, Ibibio, Jukun, etc constitute 30.3% (Table 4.2).

Table 4.3: Frequency Distribution of Respondents' Marital Status by Age

Age Group (Yrs)	Marital Status					Total
	Married	Single	Divorced	Separated	Widowed	
<20	0	1	0	0	0	1
20-29	32	1	0	0	0	33

30-39	59	5	1	1	2	68

Age Group	Educational Status					Total
40-49	42	1	2	1	6	52
50-59	42	0	0	2	10	54
Total(%)	175(84.1)	8(3.8)	3(1.4)	4(1.9)	18(8.7)	208

Most of the respondents are married (84.1%), single, divorced, separated and widowed constituted 3.8%, 1.4%, 1.9% and 8.7% respectively (Table 4.3).

Table 4.4: Frequency Distribution of Respondents' Educational Status by Age

	No formal	Primary	Secondary	Tertiary	
<20	0	0	1	0	1
20-29	1	5	11	15	32
30-39	9	10	10	40	69
40-49	6	8	13	25	52
50-59	17	2	12	23	54
Total(%)	33(15.9)	25(12.0)	47(22.6)	103(49.5)	208

Majority of the respondents have tertiary education (49.5%). Respondents with non-formal, primary and secondary education constitute 15.9%, 12.0% and 22.6% respectively (Table 4.4).

Table 4.5: Frequency Distribution of Cytological Diagnoses

Diagnosis	Frequency	Percent(%)
NILM	475	95.0
LSIL	13	2.6
HSIL	7	1.4
ASC-US	5	1.0
Total	500	100.0

Table 4.5 showed that majority (95%) were Negative for Intraepithelial Lesion of Malignancy (NILM), LSIL (2.6%), HSIL (1.4%) and ASC-US (1.0%). There were no glandular cell abnormalities observed. Plates I – III showed the morphological appearances of negative, LSIL and HSIL cervical smears respectively.

The prevalence of pre-invasive lesions on cervical smears among Family planning and Gynaecology clinic attendees in Ahmadu Bello University Teaching Hospital Zaria using the Bethesda Classification during the study period was 5%.

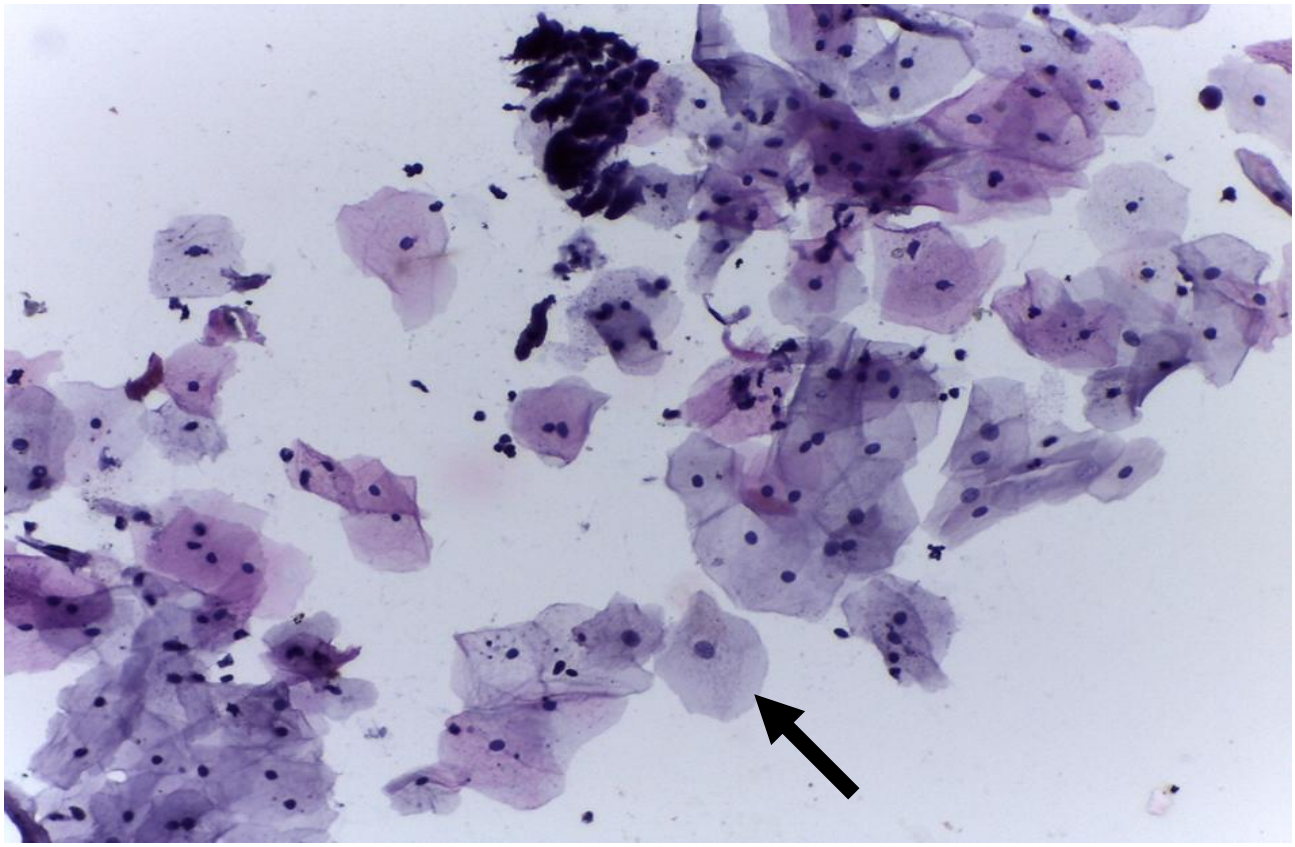


Plate I: Negative for Intraepithelial Lesion or Malignancy, NILM (Pap x100)

Note the squamous cells having regular nuclei and abundant cytoplasm (arrow). No cytological abnormality observed.

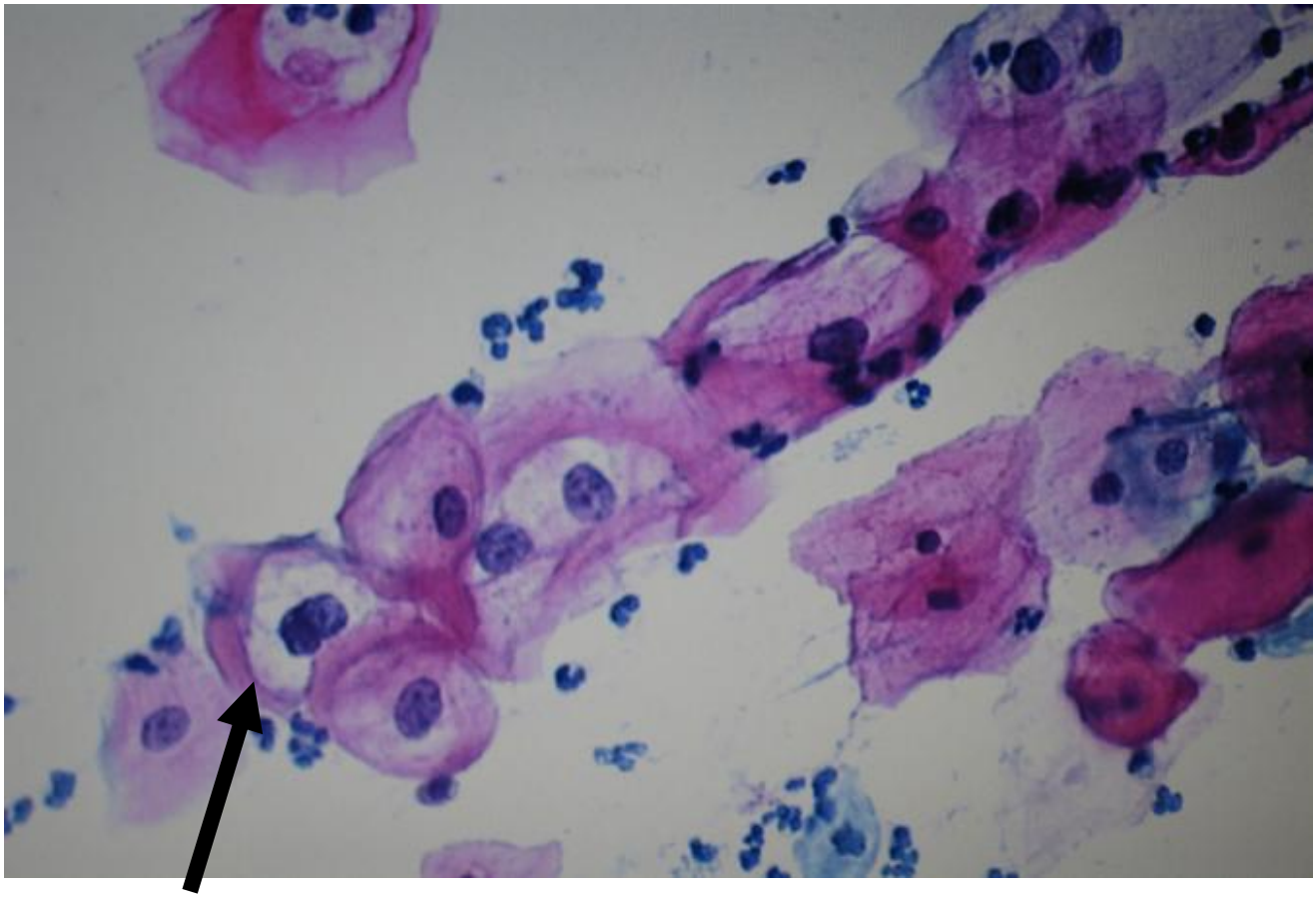


Plate II: Low Grade Squamous Intraepithelial Lesion, LSIL (Pap x200)

Note the squamous cells having mild increase in nuclear cytoplasmic ratio and koilocytotic atypia (arrow).

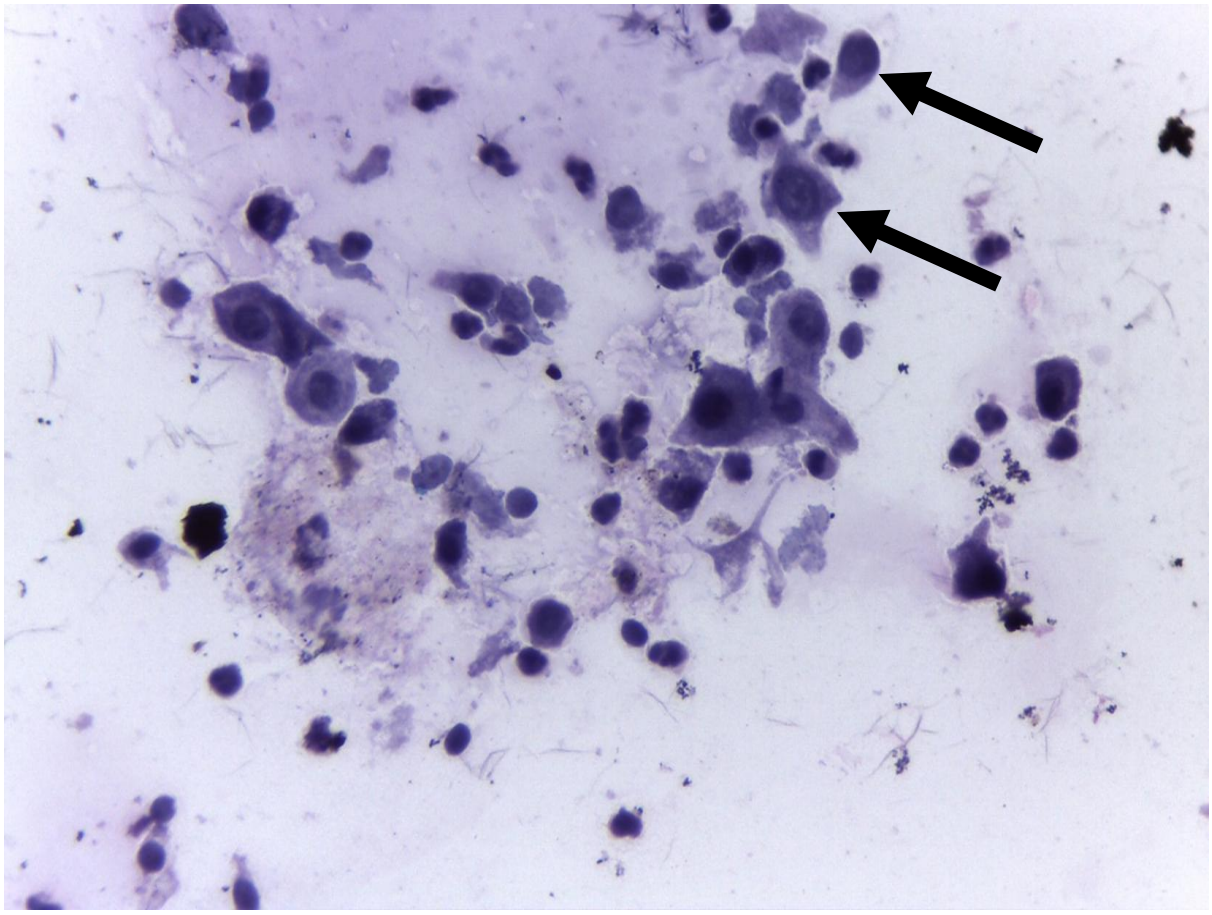


Plate III: High Grade Squamous Intraepithelial Lesion, HSIL(Pap x200)

Note the marked increase in nuclear cytoplasmic ratio, hyperchromatic nuclei and small amount of cytoplasm (arrows).

Table 4.6: Borderline (ASC-US) and LSIL cytological diagnose versus p16/Ki67 dual stain

Cytology

	Diagnosis / Biomarker Outcome	ASC-US	LSIL	Total
p16/Ki67 Stain	**Positive**	2	4	6
	Negative	3	9	12
	Total	5	13	18

Upton's "N - 1" chi-sq. = 0.131 P = 0.718

Therefore, there is no statistically significant relationship between p16/INK4a dual stain and ASC-US/LSIL cytology.

The evaluation of p16INK4a and Ki67 biomarkers using immunocytochemistry in LSIL and borderline lesions in cervical smears revealed that 6 out of the 18 cases in this category were positive and subsequently reclassified to High grade (Table 4.6). Out of the six cases, 2 were ASC-US and 4 LSIL on cytology.

ASC-US: Atypical Squamous Cells of Undetermined Significance

LSIL: Low Grade Squamous Intraepithelial Lesion

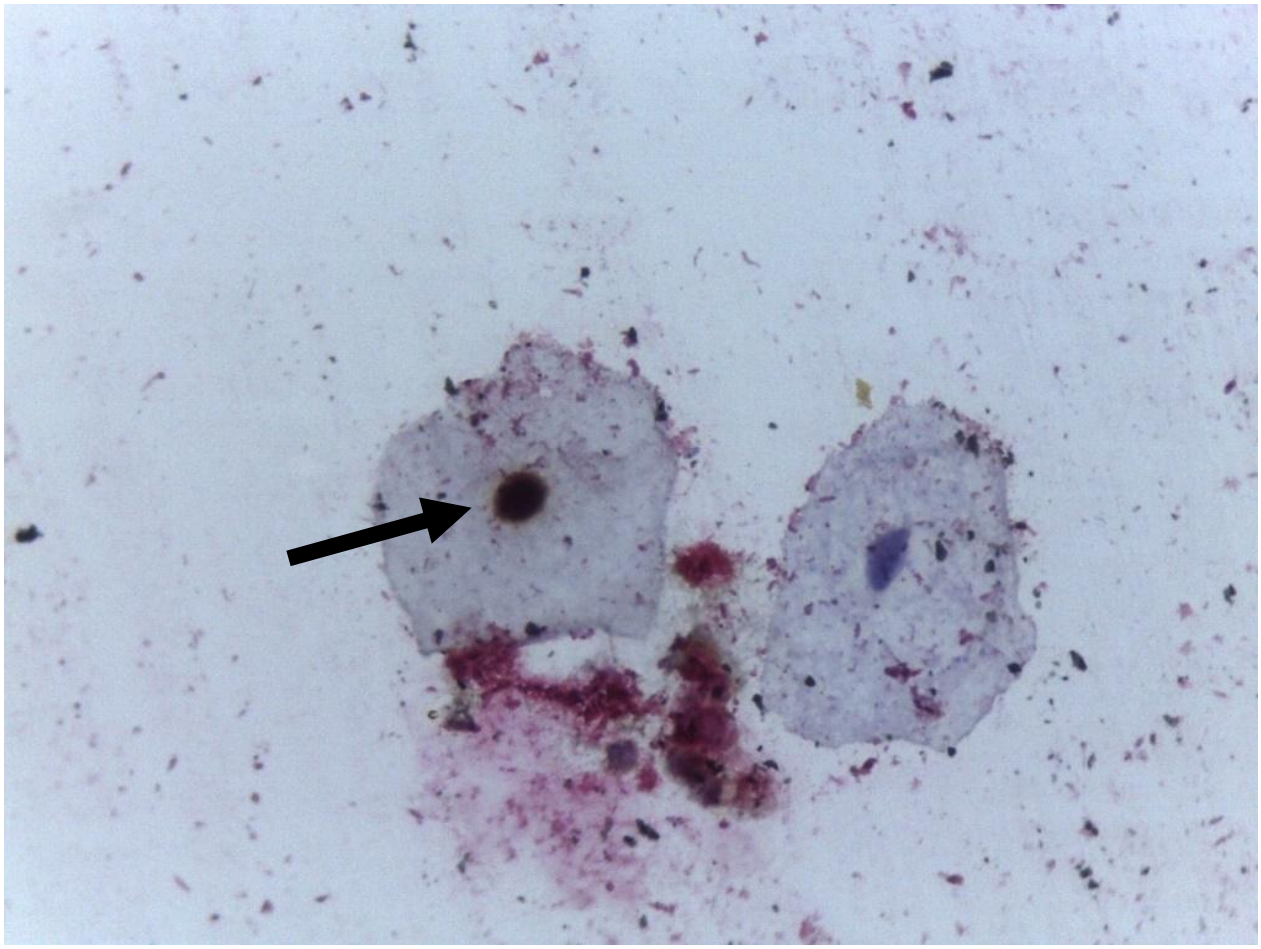


Plate IV: Negative dual stain with p16/Ki67 x400. The cell on the right (arrow) is positive for Ki-67, note the red nucleus and negative for p16INK4a. The cell on the right is negative for both p16 and Ki67.

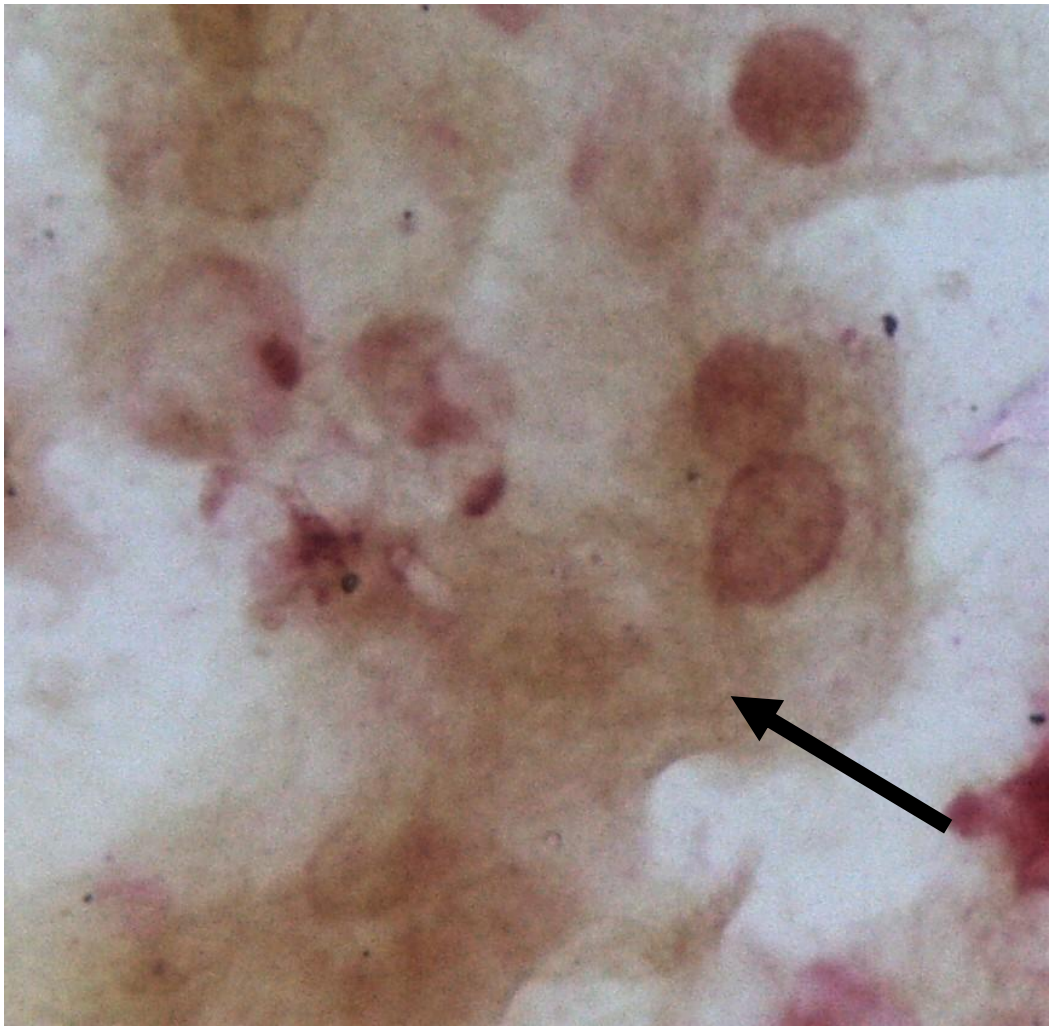


Plate V: Negative dual stain with p16 and Ki-67. However, it is positive for p16 (note the brown cytoplasm – arrow).x630

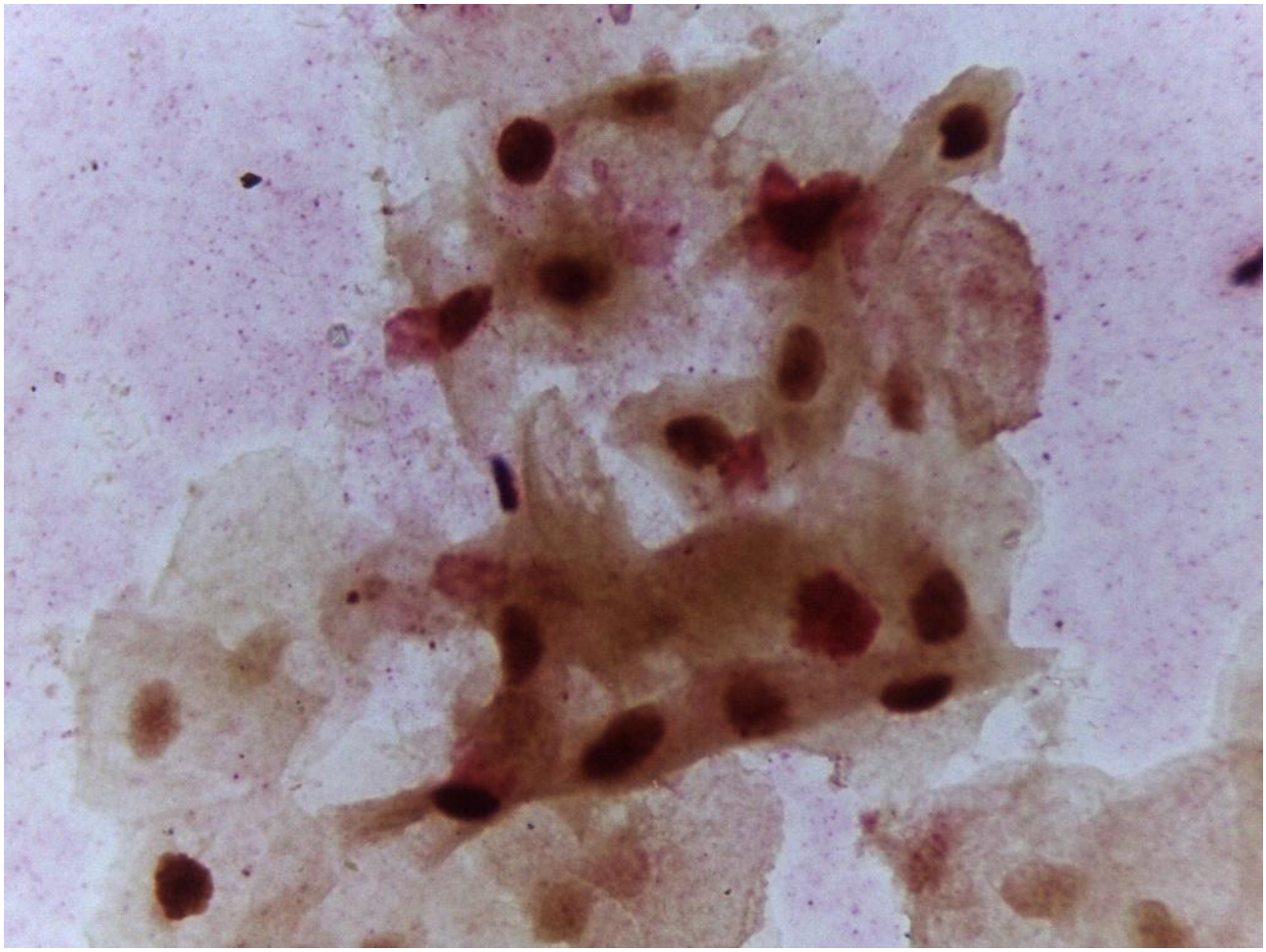


Plate VIa: Positive Dual stain with p16INK4a/Ki67 x400

Note the brown cytoplasmic staining (p6INK4a) and red nuclei (Ki67) in the same cells.

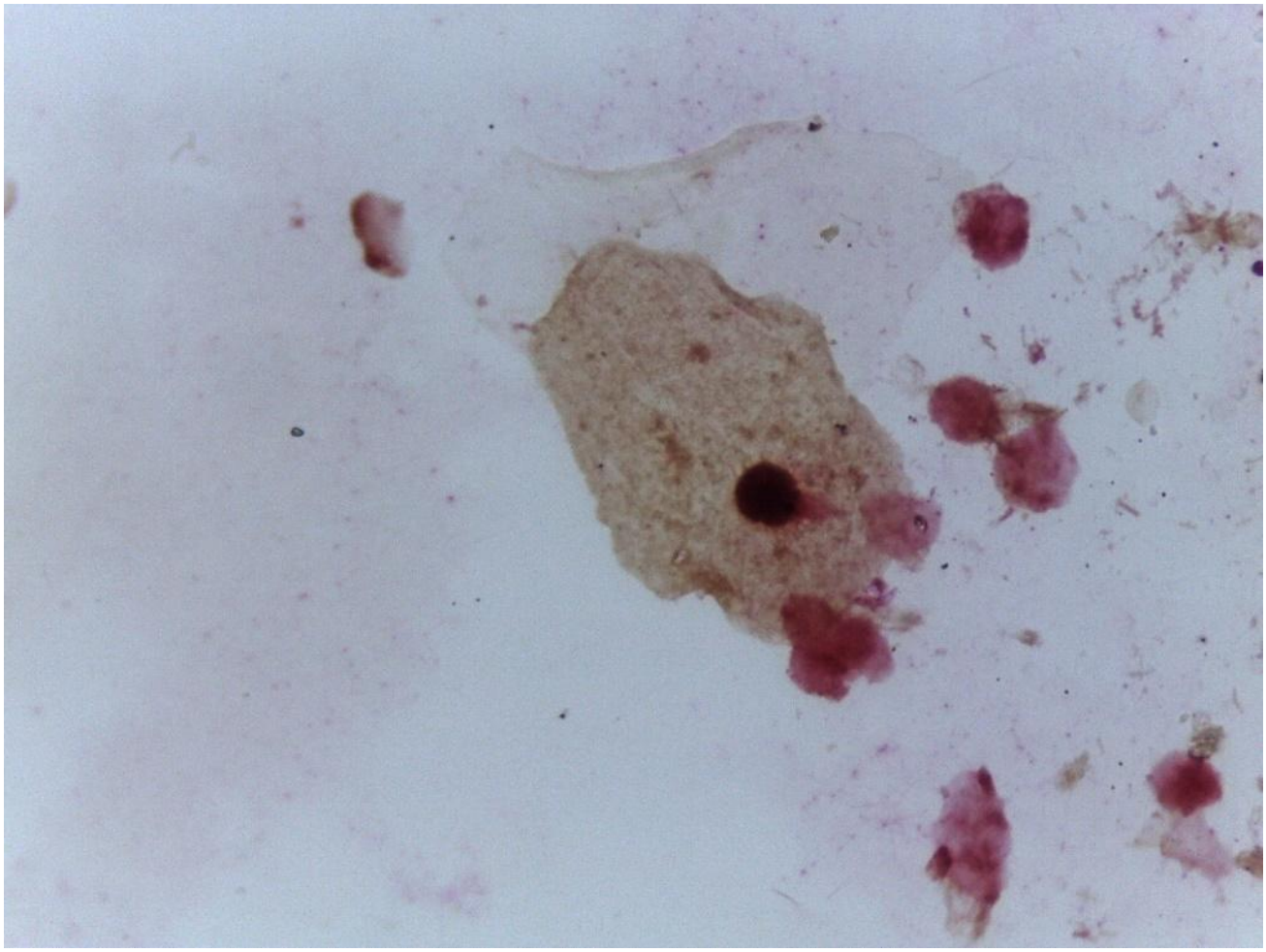


Plate VIb: Positive dual stain with p16INK4a/Ki67 x400

Note the brown staining cytoplasm (p16INK4a) and pink nucleus (ki67) in the same cell.

Table 4.7a: Characteristics of abnormal smears using cytology, p16/Ki67 and histology

Cytology	p16/Ki67	Histology
ASC-US	**Positive**	**HSIL**
LSIL	Positive	LSIL
LSIL	**Positive**	**HSIL**
LSIL	Positive	LSIL
HSIL	Negative	HSIL
HSIL	Negative	HSIL

Comparison of high grade cervical smear lesions with histological diagnosis were done in only six cases. The report showed good concordance with one case each of ASC-US and LSIL reclassified as HSIL based on histology (Table 4.7a).

Table 4.7b: Comparing HSIL and LSIL cytology with histology

		Histology		
	Diagnosis	**HSIL**	**LSIL**	**Total**
Cytology	**HSIL**	**2**	**0**	**2**
	LSIL	**1**	**2**	**3**
	Total	**3**	**2**	**5**

Table 4.7b showed correlation of cytology (HSIL & LSIL) and histology. One case of LSIL cytology reclassified as HSIL on histology. The sensitivity and specificity of cytology was 66.67% and 100% respectively.

Sensitivity = 66.67% (95% C.I. = 20.77 to 93.85%)

Specificity: 100.00% (95% C.I. = 34.24% to 100.00%)

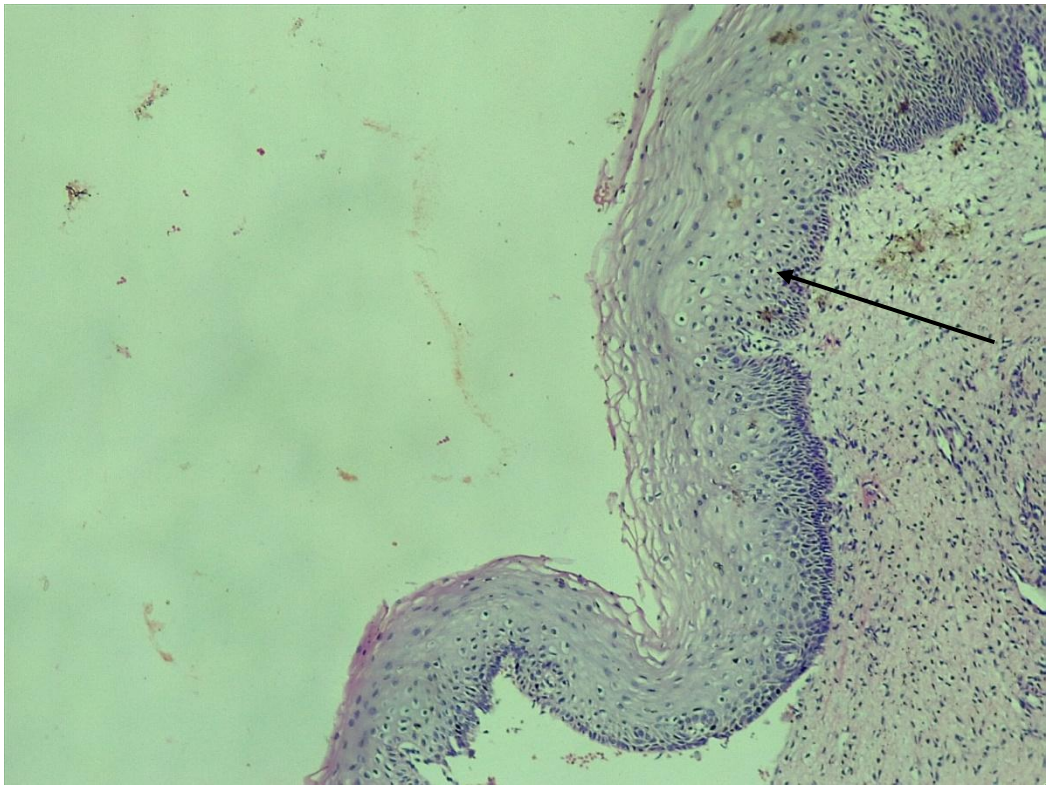


Plate VII: LSIL on Histology. H & E x100

Note the dysplasia affecting the lower one third of the ectocervix, some koilocytotic atypia (arrow) are also seen.

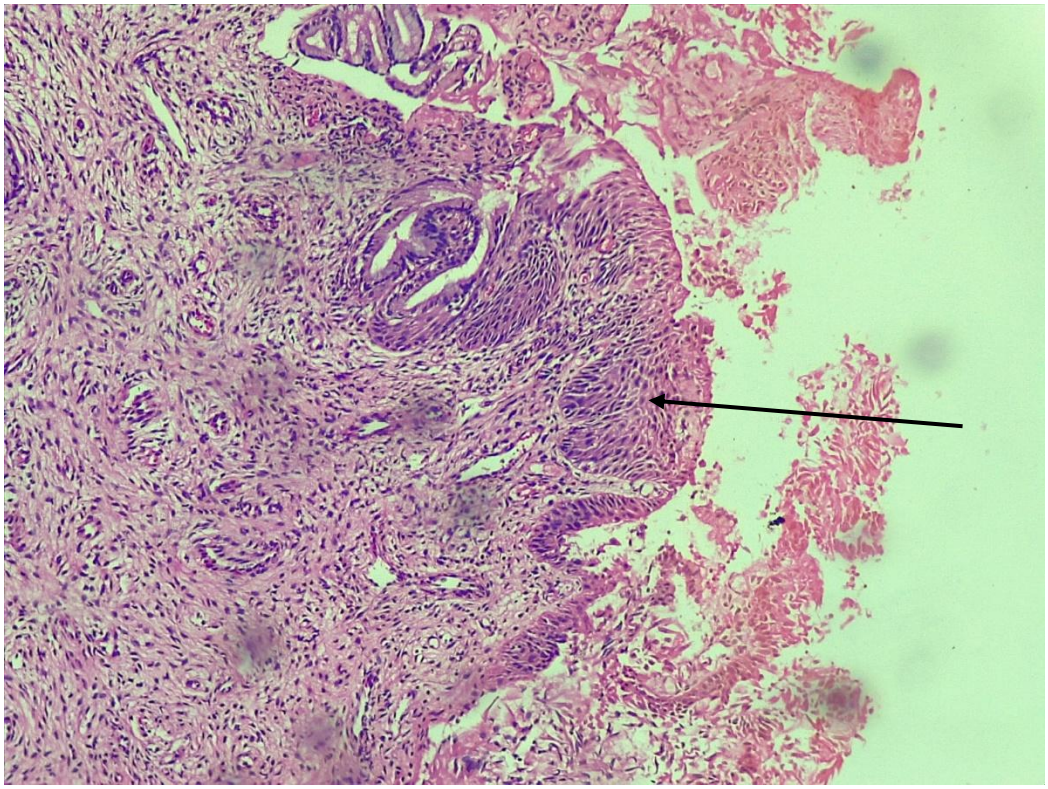


Plate VIII: HSIL on Histology. H & E x100

Note the dysplasia affecting the whole thickness of the ectocervix(arrow) in areas.

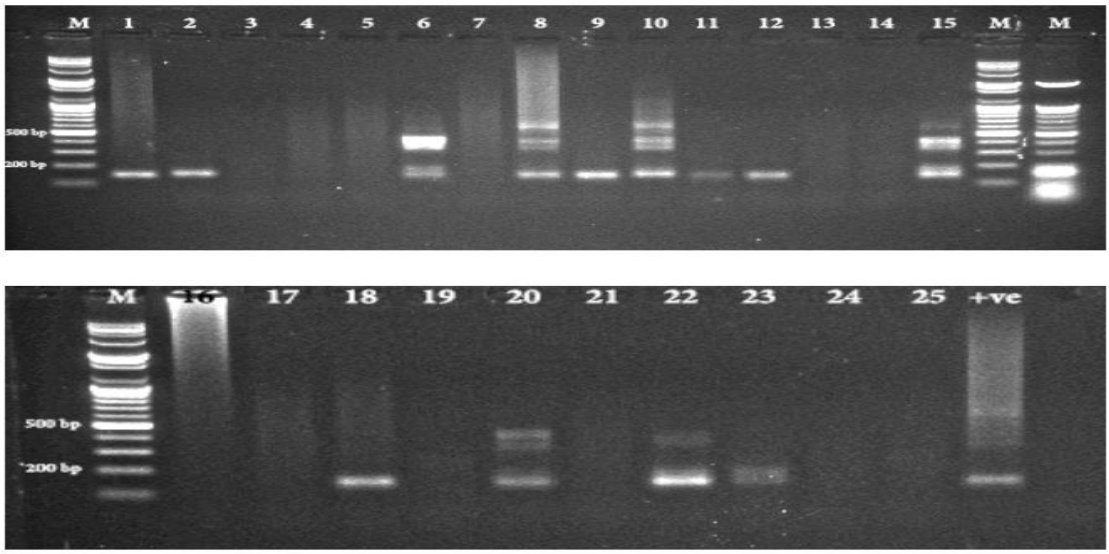


Plate IX: Gel electrophoresis of all cases with abnormal smears

Note the band at the 150bp region corresponding the L1 genomic region that is common to all Hr-HPV, presence of band at this region denote positive Hr-HPV.

The HPV DNA was positive in 13 (52%) of cases with abnormal smears using the PCR and Gel Electrophoresis (Plate VIII). Table 4.8 showed that HPV DNA was positive in 40%, 85.7% and 38.5% of ASC-US, HSIL and LSIL respectively.

Table 4.8: Frequency Distribution of HPV DNA and Cytology

	DNA		
	Positive(%)	**Negative(%)**	**Total**
ASC-US	2(40.0)	3(60.0)	5
HSIL	6(85.7)	1(14.3)	7
LSIL	5(38.5)	8(61.5)	13
Total	13	12	25

Fisher's Exact Test p value = 0.127

Table 4.8 showed that HSIL, ASCU-US and LSIL had positive HPV DNA in 85.7%, 40.0% and 38.5% respectively. There is no statistically significant relationship between Hr-HPV DNA and abnormal smears.

Table 4.9: HPV Nucleotide Sequencing Result and Genotyping

Serial Number	Specimen Number	Nucleotide sequence	HPV type
1	1	TATTTGCCGCTACATCTGCTGCTGCAGAATACAA GGCCTCTAACTTTAAGGAATTTCTGCGCCATACA GAGGAATATGATTTACAGTTTATTTTTCA	18
2	2	TGTCTGCTGCACCGAAACAGCCATACCTGCTGTA TATAGCCCTACAAAGTTTAAGGAATATACTAGGC ATGTGGAGGAATATGATTTACAGTTTATTTTTCA	31
3	6	TTATGTGCTGCCTATCTACTTCAGAACCTACATA TAAAAATACTAACTTTAAAGAGTACCTACGACAT GGGGAGGAATATGATTTACAGTTTATTTTTCA	16
4	8	ATTATGTGCTGCCATATCTACTTCAGAACCTACA TATAAAAATACTAACTTTAAAGAGTACCTACGAC ATGGGGAGGAATATGATTTACAGTTTATTTTTCA	16
5	9	TTATGTGCTGCCATATCTACTTCAGAACCTACAT ATAAAATACTAACTTTAAAGAGTACCTACGACAT GGGGAGGAATATGATTTACAGTTTATTTTTCA	16
6	10	GCTGCACCGAAACAGCCATACCTGCTGTATATAG CCCTACAAAGTTTAAGGAATATACTAGGCATGTG GAGGAATATGATTTACAGTTTATTTTTCA	33
7	11	ATACAATGTATTATGTGCTGCCATATCTACTTCA	16

		GAACCTACATATAAAAATACTAACTTTAAAGAGTACCTACGACATGGGGAGGAATATGATTTACAGTTTATTTTTCA	
8	12	TGTATTATGTGCTGCCATATCTACTTCAGAACCTACATATAAAAATACTAACTTTAAAGAGTACCTACGACATGGGGAGGAATATGATTTACAGTTTATTTTTCA	16
9	15	TTATGTGCTGCCATATCTACTTCAGAACCTACATATAAAAATACTAACTTTAAAGAGTACCTACGACATGGGGAGGAATATGATTTACAGTTTATTTTTCA	18
10	18	ATTATGTGCTGCCATATCTACTTCAGAACCTACATATAAAAATACTAACTTTAAAGAGTACCTACGACATGGGGAGGAATATGATTTACAGTTTATTTTTCA	16
11	20	TTTGTGTGCCCTGCAACATCTGGTGATACATATACAGCTGCTAATTTTAAGGAATATTTAAGACATGCTGAAGAATATGATTTACAGTTTATTTTTCAG	35
12	22	CTTTTGCACACAAGTAACTAGTGACAGTACATATAAAAATGAAAATTTTAAAGAATATATAAGACATGTTGAAGAATATGATTTACAGTTTATTTTTCATG	33
13	23	TTGTGTGCTACAGCATCCACGCAGGATAGCTTTAATAATTCTGACTTTAGGGAGTATATTAGACATGTGGAGGAATATGATTTACAGTTTATTTTTCA	52

Table 4.10: Frequency Distribution of HPV genotypesin abnormal smears

HPV Genotype	Frequency	Percent(%)
16	6	46.2
18	2	15.4
33	2	15.4
31	1	7.7
35	1	7.7
52	1	7.7
Total	13	100.0

The High risk HPV genotyping using sequencer showed 5 different types – type 16, 18, 31. 33, 35 and 52. Type 16 is the most prevalent in 46.2% of cases, followed by Types 18 and 33 with 15.4% of cases each.

The sequence chromatographs are shown in Plates Xa and Xb.

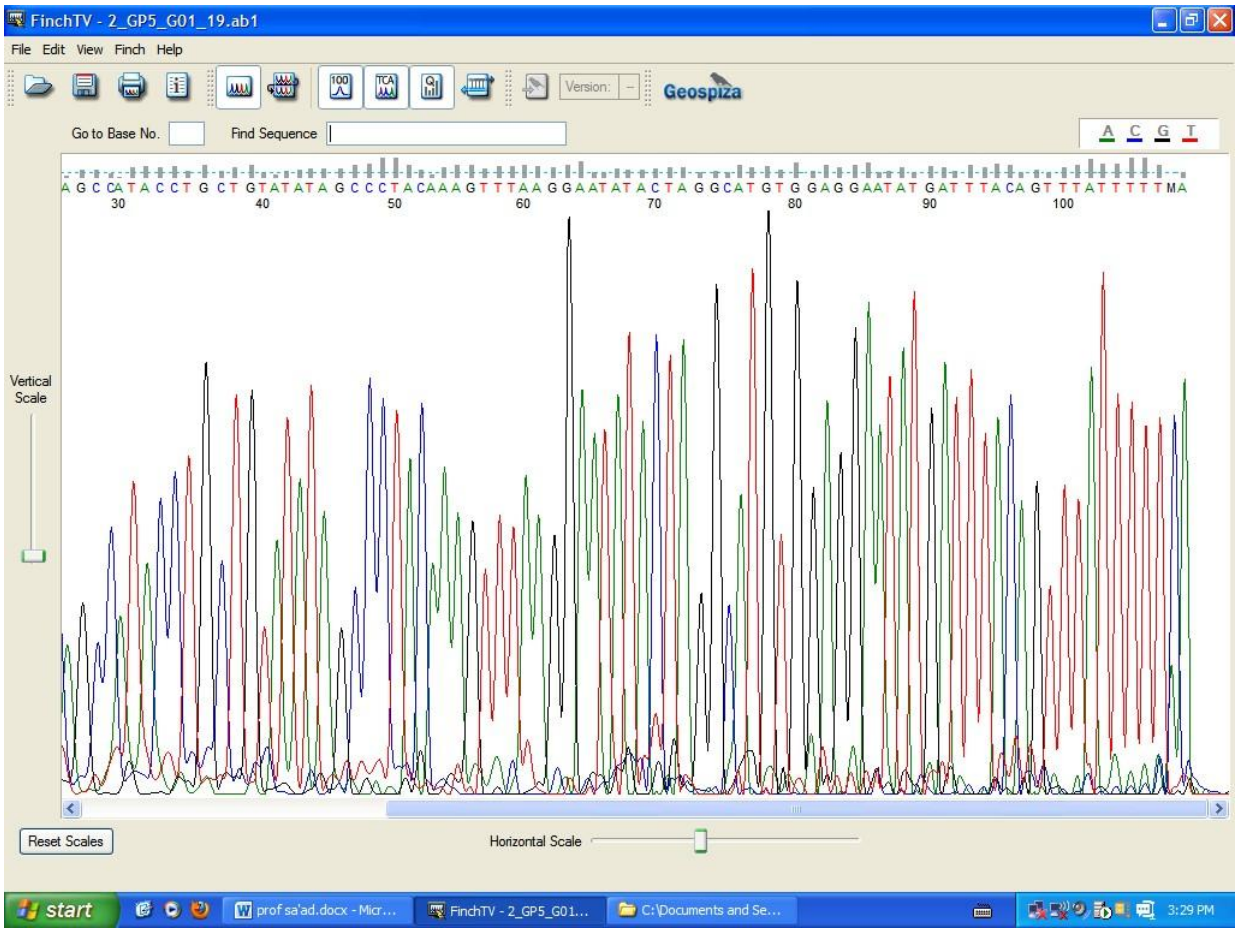


Plate Xa: Sequence chromatograph

Note the sequence alignments of the bases in the chromatograph against HPV genome database in GenBank database by online BLAST analysis real time.

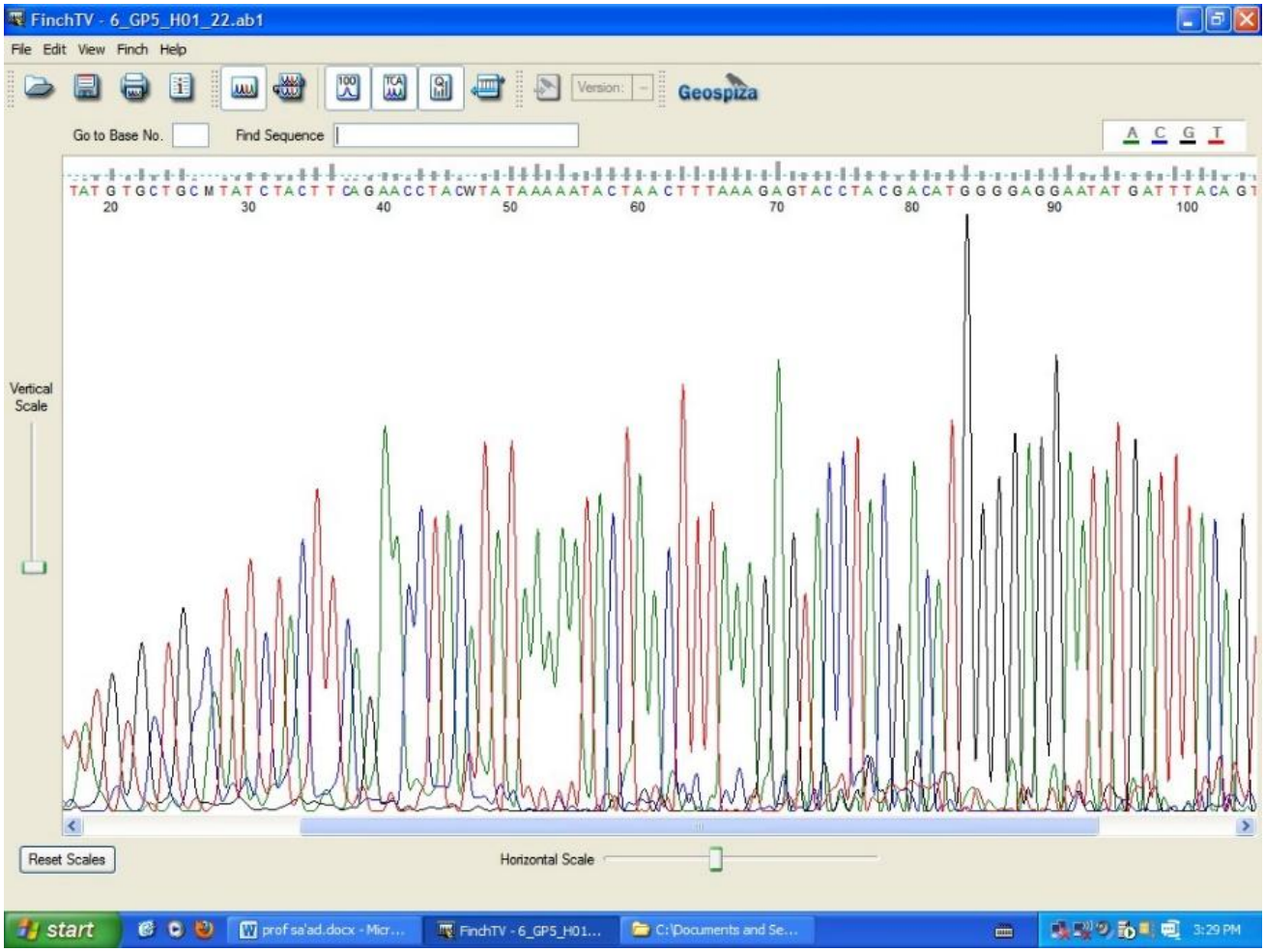


Plate Xb: Sequence chromatograph

Note the sequence alignments of the bases in the chromatograph against HPV genome database in GenBank database by online BLAST analysis real time.

Table 4.11: Bivariate Analysis of some Risk Factors associated with abnormal cytology

Variable	Cytology		Odds ratio (95% CI)	P- Value
	SIL (%)	Negative (%)		
Age (year)n=500				
<50	20(4.0)	369(73.8)	1.149 (0.421, 3.134)	0.786
≥50	5(1.0)	106(21.2)		
Education (n=208)				
Low (< secondary)	4(1.9)	54(26.0)		
High (secondary & above)	10(4.8)	140(67.3)	1.037 (0.312, 3.448)	1.000
Parity (n=166)				
High (4+)	2(1.2)	107(64.5)	0.134 (0.027, 0.666)	***0.008**
Low (<4)	7(4.2)	50(30.1)		
Age at first marriage (n=198)				
Inappropriate (<18yrs)	3(1.5)	59(29.8)	0.641 (0.170, 2.415)	0.758
Appropriate (18+yrs)	10(5.1)	126(63.6)		
Age at first sex (n=212)				
<18	4(1.9)	63(29.7)	0.857 (0.259, 2.839)	1.000
18+	10(4.7)	135(63.7)		
Uncircumcised partner (n=202)				
Yes	2(1.0)	17(8.4)	1.676 (0.346, 8.121)	0.626
No	12(5.9)	171(84.7)		
Oral contraceptives(n=211)				
Yes	6(2.8)	72(34.1)	1.302 (0.435, 3.902)	0.637
No	8(3.8)	125(59.2)		

Multiple sexual partners(n=205)				1.000
Yes	2(1.0)	36(17.6)	0.788 (0.167, 3.711)	
No	11(5.4)	156(76.1)		
Partner with multiple previous sexual partners(n=187)				0.548
Yes	3(1.6)	62(33.2)	0.542 (0.144, 2.043)	
No	10(5.3)	112(59.9)		
Immunosuppressive disease (n=206)				0.994
Yes	0(0.0%)	8(3.9)	0.749 (0.0411, 13.630)	
No	14(6.8)	184(89.3)		
Cigarette smoking (n=210)				0.397
Yes	0(0.0)	2(1.0)	2.683 (0.123, 58.530)	
No	14(6.7)	194(92.4)		
Sexually Transmitted Diseases (n=208)				
Yes	1(0.5)	106(51.0)		
No	13(6.3)	88(42.3)	0.064 (0.008, 0.498)	***0.001**
Marital status (n=208)				
Unmarried	1(0.5)	32(15.4)		
Married	13(6.3)	162(77.9)	0.389 (0.049, 3.083)	0.703

These associated risk factors like age, level of education, parity, age at first sexual intercourse, multiple sexual partners, uncircumcised male partner etcwere all found to be within the 95% Confidence Interval but only parity and STDs were found to be statistically significant ($P<0.05$)

CHAPTER FIVE

5.0 DISCUSSION

Cervical cancer is one of the most common cancers in developing countries despite a favourable natural history that allows for early detection of preinvasive lesions. Pap smear cytology is one of the widely used screening methods however, its limitation particularly on borderline cases led to the introduction of evaluation using HPV DNA testing as well as molecular biomarkers.

This study was conducted among five hundred (500) women who attended the Family Planning and Gynaecology Clinics of the Ahmadu Bello University Teaching Hospital Zaria and most of the respondents were in the fifth decade of life (Table 4.1). The most prevalent ethnic group in the study was Hausa, followed by Yoruba and Ibo as shown in Table 4.2. This is due to the fact that even though Zaria is cosmopolitan with a mix of different ethnic groups, it is largely a Hausa dominated community (King, 2003). Table 4.3 showed that majority of the respondents were married in 84.1% and most of them have some form of formal education as 49.5% had tertiary education. This may be explained due to the fact that there is a direct relationship between level of education and health-seeking behaviour. (Musoke*et al*., 2014)

The prevalence of pre-invasive cervical lesions on smears among the study participants using the Bethesda System (TBS) was 5% (Table 4.45. The result of this study is similar to a

previous study in Zaria which reported 7% (Avidime*et al*., 2014). It is also similar to studies in Romania (Stolnicu*et al*., 2014), Sudan (Awad, 2016) and Rwanda (Makuza*et al*., 2015) with prevalence of 5.9%, 3.5% and 5.9% respectively. However, the result is at variance with reports from Minna – Nigeria (Mohammed, 2014), Ethiopia (Getinet*et al*., 2015) and Jalingo – Nigeria (Ahmed *et al*., 2013) with prevalence of 13%, 14.1% and 16.7% respectively. The reason for this disparity may be due to the fact that the sample size in these studies were relatively small and may be responsible for the higher prevalence noted in their studies.

The cytological diagnoses showed that majority (95%) were Negative for Intraepithelial Lesion or Malignancy (NILM). Those with abnormal smears showed LSIL (2.6%), HSIL (1.4%) and ASC-US (1.0%) (Plates I-III). Cervical Pap smear screening in women in the US is instructedto commence three years after initiation of sexual activity, andno later than age 21 years. Of the 50 million cervical Pap smearsdone annually in the US, about 3–4% are reported as LSIL,0.6% as HSIL and 4–6% as ASCUS. In recent years, high-risktype HPV testing has been used to guide the management ofabnormal cervical Pap smears (Ibeanu, 2011).

The ASCUS/SIL ratio is 1:4 in this study which showed good accuracy in making diagnosis or it may also be due to the small number of abnormal smears seen in this study.The most commonly made abnormal diagnosis on a Papanicolaou smear is “Atypical Squamous Cells of Undertermined Significance” (ASCUS). The authors of TBS recognized the potential overuse of the ASCUS diagnosis and suggested a benchmark of ASCUS/SIL ratio of 3:1. However, there is no general consensus on an ideal ASCUS/SIL ratio (Juskeviciu*set al*., 2001).

In the evaluation of LSIL and borderline lesions, 6 out of 18 cases (33.3%) were positive for the dual immunocytochemical stain with p16INK4a/Ki67 and subsequently reclassified to

high grade (Table 4.6; Plates IV,V,VIa and VIb). However, this is found not to be statistically significant (P>0.05).The advantage of p16/Ki-67 dual staining is that it does notrequire morphological interpretation. This is an improvement ofthe evaluation of p16 alone, which is still morphology dependent. However, a trained pathologist will also beinfluenced by the morphology as it is their expertise. Therefore,in contrast to the HPV test, the evaluation of the dual staining isnot totally objective (Uijterwaal*et al*., 2014). Dual stain with p16/Ki67 can be done on both de-stained pap smear slide or on liquid based cytology material.

The overexpression of p16 in cervical dysplasia has been shown to be associated with the transforming activity of the E7 oncoprotein of high-risk HPV types and can be regarded as a surrogate marker of the E7-mediated inactivation of the tumor-suppressor function of the retinoblastoma protein (pRb). There is experimental evidence that p16 overexpression is induced by the abrogation of a negative feedback mechanism that is mediated by functional pRb at the transcriptional level under normal physiological conditions. Functional inactivation of pRb, though, may lead only to genetic instability and, thus, malignant transformation when it occurs in DNA replication-competent cells. Therefore, simultaneous detection of p16 overexpression and expression of the proliferation marker Ki-67 within the same cervical epithelial cell should indicate deregulation of the cell cycle (Samarawardana*et al*., 2010). Because under normal physiological conditions, the simultaneous expression of a protein with tumor-suppressive function (ie, p16) and a proliferation marker (such as Ki-67) should mutually exclude each other, it is now possible to use the immunocytochemical detection of p16/Ki-67 coexpression to identify cells with deregulated cell cycle in cervical cytology specimens, independent from morphology-based interpretation parameters. The presence of 1 or more double-immunoreactive cells may be used as an indicator of underlying HSIL (Schmidt *et al*., 2011).

The comparison of high grade cervical lesions on cytology with histological diagnosis were done and there was good concordance. The correlation of HSIL and LSIL cytology with histology showed sensitivity and specificity of 66.67% and 100% respectively (4.7a and b; Plates VII-VIII). However, this should be interpreted with caution as this was carried out on a small number of cases.Despite our small number of cases, Saha and Thapa (2005) found the sensitivity and specificity of cytology in LSIL and HSIL to be 83.3% and 86.4% respectively is similar to ours. This is also similar to the works of Rathod and Singla (2015) in India.

The prevalence of Hr-HPV in the abnormal smears was 52% (Plate IX) which is similar to 50% from a study in Baltimore – USA (Rositch*et al*., 2013) and 64.5% from Loja – Ecuador (Aguilar *et al*., 2017). However, this contrasts previous works in Taipei – Taiwan (Sun *et al*., 2005) and Victoria – Australia (Stevens *et al*., 2009with prevalence of 74.4% and 83.9% andrespectively.The reason for this difference may be due to the relatively larger sample sizes of these studies. A previous work in Zaria by Aminu*et al*. (2007) showed a prevalence of 88.0%, however their work was ELISA-based and not PCR-based like ours.This is shown on Plate IX.

There was a positive correlation between the Hr-HPV DNA and cytology in the abnormal smears which showed positivity in 40%, 85.7% and 38.5% of ASC-US, HSIL and LSIL cases respectively (Table 4.9). However, this is not statistically significant (P>0.05). It has been noted that HPV DNA testing has agreater sensitivity for the detection of high grade lesions comparedto cytology after a single screening round. Compared to cytology, Pileggi*et al.* (2017) found that HPV DNA testing is ableto detect 45% and 48% more cases of CIN2 and CIN3,respectively, in all age groups.Detection of HPV DNA by PCR is exquisitely sensitive and specific and is used in certain settings. However, again results can prove misleading as DNA detection can indicate the presence of viral sequences but not whether the virus is actively expressing the proteins that cause disease (Graham, 2010).Luttmer*et al*. (2016)

reported that women who test positive for a high-risk type of the human papillomavirus (HPV) require triage testing to identify those women with high grade lesions or cancer (≥CIN3). Although Pap cytology is considered an attractive triage test, its applicability is hampered by its subjective nature.

Molecular genotyping of the Hr-HPV seen in the abnormal smears showed five different types – 16, 18, 31, 33, 35 and 52 (Table 4.9, Table 4.10a, Table 4.10b; Plate Xa, Plate Xb). Our report is slightly similar to reports from Gombe (Manga *et al*. 2015) which showed the following genotypes in order of frequency - HPV types 18 and 16 were the most predominantwith proportions of 44.7 % and 13.2 % respectively; multiple HPV infections were identified among 7.9 % ofthe specimens. No case of co-infection was observed in this study. Other genotypes identified were types 31,33, 35, 38, 45, 56, 58, 82 and KC5 in varying proportions. However, reports by Kolawole*et al.* (2015) in Lokoja - Nigeria revealed HPV 35, 31,18 and 16 strains in decreasing frequency. Okwuraiwe*et al.* (2015) gave a preliminary report of Nigerian cervical cancer surveillance report and concluded that theotherhighriskgenotypesaremoreprevalent(9.4%)thanHPV16and18 thataregloballyknowntocausecervicalcancerandarethetargetsforcurrentlymanufacturedvaccine s.

The infection with HPV genotypes has geographical variation. In the United States, HPV-16 was the most common type, but HPV-58 was the second most prevalent in Mexican population (Giuliano, *et al*., 1999). In Spain 75% of screened women who were sero-positive for HPV-58 (Touze, *et al*., 2001). HPV-58 was the second most common genotype in Japan (Sasagawa, *et al*., 2001). The association between cervical cancer and high risk oncogenic types of HPV is clearly demonstrated (Bosch, *et al*., 1995). Women with cervical intraepithelial neoplasia III and invasive cervical cancer have high prevalence of HPV- 16. HPV-18 (Rabelo-Santos *et al*., 2003). Huwanga*et al*. (2004) reported that HPV-18 was

present more frequently in (84.6%) adenocarcinomas and adenosquamous carcinomas. In Mali, HPV DNA was identified in 97% of cervical cancer cases, and HPV types 16, 18, and 31 were detected in 60% of cases and 45% of controls (Bayo*et al*., 2002).

Table 4.11 showed some risk factors for cervical cancer beinganalysed and they include: age, educational status, parity, age at first marriage, age at first sexual intercourse, uncircumcised male partner, use of oral contraceptives, multiple sexual partners, sexual partners with multiple previous partners, immunosuppressive diseases, cigarette smoking, sexually transmitted diseases and marital status. These were all found to be associated with preinvasive lesions of the cervix but only parity and sexually transmitted diseases were found to be statistically significant ($P<0.05$).

The central etiologic factor for the development of cervicalcancer is persistent infection with high-risk oncogenic HPV types (zurHausen, 2009). Other recognized risk factors for cervical cancer arerelated to the sexual acquisition of HPV as well as immune dysfunction,exposure to mutagens and hormonal factors. Studiesin twins suggest that genetic background typically plays a smallrole in the development of cervical cancer. The most notablerisks are early age of sexual activity, multiple sexual partners,exposure to other sexually transmitted diseases, cigarette smoking,oral contraceptive use, human immunodeficiency virusinfection and immunosuppressive drug therapy (Ibeanu, 2011). Poor accessto cancer screening services as well as non-compliance withscreening visits are social risk factors related to lower socioeconomicstatus and lower educational levels that are observedmore frequently among African American, American Indianand Hispanic women compared to Caucasians in the United States. Unfortunately, these same factors negatively impactHPV vaccine implementation in these populations, given itscurrent high cost(Ibeanu, 2011).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study showed that pre-invasive lesions of the cervix is not uncommon as detected by cytology. The dual immunocytochemical stain with p16/Ki67 on LSIL and borderline cases has shown an encouraging result and the need for incorporation as a triage tool. High grade lesions revealed a high prevalence of HPV DNA and revealed types 16, 18 and 33 being the most common seen in the study.

6.2 RECOMMENDATIONS

Following the outcome of this research work, the following recommendations were made:

a. Vaccination against HPV types 16, 18 and 33 should be considered for the at-risk population.
b. Mass screening for cervical cancer should be encouraged by the state and federal governments to come up with reliable prevalence from the community.

c. Dual immunocytochemical stain with p16/Ki67 should be considered in triage for LSIL and borderline lesions of the cervix.
d. Larger studies across different parts of Nigeria are recommended to determine the actual prevalence of HPV in cervical scrapings and to determine the genotypes in order to plan for preventive services.
e. Awareness campaigns targeting risk factors for cervical cancer by the state and federal governments are encouraged.

6.3 CONTRIBUTIONS TO KNOWLEDGE

The following are the contribution of this research work to body of knowledge:

1. The prevalence of abnormal cervical smears was 5% among women attending Family Planning and Gynaecology Clinics of ABUTH Zaria
2. A total of 33.3% of ASCUS/LSIL cases were positive for p16INK4a/Ki-67 dual immunocytochemical stain and were subsequently reclassified to High grade lesions
3. The sensitivity and specificity of cytology was found to be 66.7% and 100.0% respectively
4. The prevalence of Hr-HPV DNA in abnormal smears was found to be 52%
5. Hr-HPV genotyping revealed types 16, 18, 31, 33, 35, and 52
6. Risk factors such as parity and sexually transmitted diseases were found to be associated with preinvasive lesions of the cervix ($p \leq 0.05$).

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APPENDIX I

PAPANICOLAOU STAINING METHOD

Papanicolaou staining technique:

1. The smears were removed from the fixative
2. Rinsed in descending grades of alcohol (80%, 70% and 50%) and in water for 10seconds each

3. Stained in Harris Heamatoxylin for 2 minutes each
4. Rinsed in water for 1-2 minutes each
5. Differentiated in 1% acid alcohol for a few seconds until only the nuclei retained the stain
6. Washed and blued in tap water for 3 seconds each
7. Transfered to 70% alcohol for a few seconds each
8. Transfered to 95% alcohol for a few seconds each
9. Stained in Orange Green 6 for 2 minutes each
10. Rinsed in two changes of 95% alcohol
11. Stained in Eosin Azure 50(EA 50) for 2 minutes each
12. Rinsed in two changes of 95% alcohol
13. Dehydrated in absolute alcohol
14. Cleared in two changes of xylene for two minutes each
15. Mounted using DPX and covered with cover slips (24 x 50 mm cover slips.).
16. The smears were examined using a light microscope.

APPENDIX II

BETHESDA CLASSIFICATION

INTERPRETATION/RESULT

NEGATIVE FOR INTRAEPITHELIAL LESION OR MALIGNANCY,
(when there is no cellular evidence of neoplasia, state this in the General Categorization above and/or in the Interpretation/Result section of the report, whether or not there are organisms or other non-neoplastic findings).

- ORGANISMS:
  * Trichomonas vaginalis
  * Fungal organisms morphologically consistent with Candida spp
  * Shift in flora suggestive of bacterial vaginosis
  * Bacteria morphologically consistent with Actinomyces spp.
  * Cellular changes consistent with Herpes simplex virus

- OTHER NON NEOPLASTIC FINDINGS (Optional to report; list not inclusive):
  * Reactive cellular changes associated with
    - inflammation (includes typical repair)
    - radiation
    - intrauterine contraceptive device (IUD)
  * Glandular cells status post hysterectomy
  * Atrophy

OTHER

- Endometrial cells (in a woman >= 40 years of age)

(Specify if 'negative for squamous intraepithelial lesion')

EPITHELIAL CELL ABNORMALITIES

- SQUAMOUS CELL
  * Atypical squamous cells
    - of undetermined significance (ASC-US)
    - cannot exclude HSIL (ASC-H)
  * Low grade squamous intraepithelial lesion (LSIL)
  (encompassing: HPV/mild dysplasia/CIN 1)
  * High grade squamous intraepithelial lesion (HSIL)
  (encompassing: moderate and severe dysplasia, CIS, CIN 2
  and CIN 3)
    - with features suspicious for invasion (if invasion
      is suspected)
  * Squamous cell carcinoma

- GLANDULAR CELL
  * Atypical
    - endocervical cells (not otherwise specified (NOS) or specify in comments),
    - endometrial cells (NOS or specify in comments),
    - glandular cells (NOS or specify in comments)
  * Atypical
    - endocervical cells, favor neoplastic
    - glandular cells, favor neoplastic
  * Endocervical adenocarcinoma in situ
  * Adenocarcinoma:
    - endocervical
    - endometrial
    - extrauterine
    - not otherwise specified (NOS)

OTHER MALIGNANT NEOPLASMS: (specify)

APPENDIX III

HISTOLOGICAL TECHNIQUE

Gross and histological processing of surgical specimens were performed according to standardised surgical pathology protocols, histological diagnoses were made using the WHO classification. The histological techniques (Bancroft and Stevens, 1999) involve:

a) Tissue fixation in 10% neutral buffered formalin

b) Gross examination and tissue sectioning

c) Tissue dehydration using graded ethanol concentration (70%, 95%, 100%)

d) Tissue clearing with xylene

e) Paraffin wax embedding

f) Microtomy trimming and sectioning at 5µ

g) Floating out tissues in water bath

h) Section drying on hot plate

HAEMATOXYLIN AND EOSIN STAINING PROTOCOL

a) Dewax sections, rehydrate through graded concentrations of ethanol (100%, 95%, 70%)

b) Stain in Harris Haematoxylin for 5 – 15mins

c) Wash well in running tap water until sections blue for five minutes or less

d) Differentiate in 1% acid alcohol for 5 – 10 secs

e) Wash well in tap water until sections are again blue for 5mins or less

f) Stain in Eosin for 10mins

g) Wash in running tap water for 1 – 5mins

h) Dehydrate through alcohols, clear and mount.

APPENDIX IV

IMMUNOCYTOCHEMICAL DUAL STAINING WITH p16/Ki67

Immunocytochemical stains comprising Ki67 (Biorbyt®, UnitedKingdom) and p16INK4a (DBBiotech®, Slovakia) will be done on liquid based cytology specimens using the protocol outlined below as shown in Appendix IV:

1. Smear made on charged glass slide
2. Block smear in 3% Hydrogen peroxide for 10 minutes.
3. Wash in TBS
4. Treat with epitope retrieval buffer (pH 6) at 95˚C for 20 minutes
5. Wash in TBS.
6. Incubate in anti-P16 (DBBiotech®, Slovakia) for 60 minutes.
7. Wash in TBS.
8. Incubated smear in Mouse + Rabbit HRP (DBBiotech®, Slovakia) for 30 minutes.
9. Wash in TBS
10. Treat smear with DAB + Chromogen Substrate (DBBiotech®, Slovakia) for 7 minutes.
11. Wash in distilled water.
12. Incubate smear in alkaline phosphatase anti-Ki67 (Biorbyt®, United Kingdom) for 60 minutes.
13. Wash in TBS
14. Treat smear with Permanent Red + Substrate (Cellmarque®, U.S.A.) for 5 minutes.
15. Wash in distilled water.
16. Counterstain in MayersHaematoxylin for 2 minutes.
17. Air-dry and mount with DPX
18. Review Positive and Negative Controls and report the tested specimens.

APPENDIX V

DNA extraction using AccuPrep® Genomic DNA Extraction Kit

1. 20 µl of Proteinase K was added to a clean 1.5 ml tube.

2. 200 µl of sample was added to the tube containing Proteinase K.

3. 200 µl of GB Buffer was added to the sample and mix immediately by vortex mixer.

4. Incubation at 60℃ for 10 min was carried out.

5. 400 µl of absolute ethanol was added and mixed well by pipetting.

After this step, sample was spun down to get the drops clinging under the lid.

6. Carefully transferred the lysate into the upper reservoir of the Binding column tube (fit in a collection tube) without wetting the rim.

7. Close the tube and centrifuge at 8,000 rpm for 1 min. Binding column tube was closed to avoid aerosol formation during centrifugation. Where lysate has not completely passed through the column after centrifugation, we centrifuged again at a higher speed (>10,000

rpm) until the binding column tube was empty.

8. Discard the solution from the collection tube into a disposal bottle and reuse the collection tube.

9. Add 500 µl of WA1 Buffer without wetting the rim, close the tube, and centrifuge at 8,000 rpm for 1 min.

Detection and typing of HPV

All the cervical specimens were tested for the presence of oncogenic HPV DNA using a nested Polymerase Chain Reaction (nPCR) with GP5+/GP6+ (GP5+[5′ TTTGTTACTGTGGTAGATACTAC-3′] and GP6+ [5′-GAAAAATAAACTGTAAATCATATTC-3′]) and PGMY 09/11 consensus primers which

amplifies a 150 bp fragment of the L1 HPV genomic region. Accu-Power HotStart Premix (Bioneer Corporation, South Korea) was used for the PCR. Genotypic identification was achieved by direct sequencing using the Gp 6+ oligoprimer. Thermal cycler (Bio Rad) and Sequencing machine (Beckman Coultier CEQ 2000XL) were used for PCR and sequencing respectively. Sequence alignments wasperformed against various standard HPV genotype sequences stored in the GenBank database by on-line BLAST analysis to arrive at specific genotyping.

APPENDIX VI

QUESTIONNAIRE

DEPARTMENT OF PATHOLOGY

AHMADU BELLO UNIVERSITY, ZARIA

REPRODUCTIVE HEALTH PARAMETERS OF WOMEN ATTENDING FAMILY PLANNING AND GYNAECOLOGY CLINICS OF ABUTH ZARIA.

NOTE:ALL information given in the questionnaire shall be treated with strict confidentiality

Tick the boxes provided as appropriate

A. SOCIO-DEMOGRAPHIC DATA

1. Age----------
2. Ethnic group: Hausa [ ] Igbo [ ] Yoruba [ ] Others: ----------
3. Marital status:Married [ ] Divorced [ ] Single [ ] Separated [ ] Widowed [ ]
4. Religion: Islam [ ] Christianity [ ] Others: ----------
5. Educational status: No formal education [ ] Primary [ ] Secondary [ ] Tertiary [ ]
6. Parity----------
7. Age as at first marriage---------- (for married women)

B. REPRODUCTIVE HEALTH PARAMETERS

Which of the following factors applies to you? Tick the appropriate box please.

1. Have you had sexual intercourse before <18 yrs? Yes [ ] No [ ]
2. Do you have (or had) multiple sexual partners? Yes [ ] No [ ]
3. Does your husband have other wife (or wives)? Yes [ ] No [ ]
4. Does your male partner have multiple previous sexual partners: Yes [ ]No [ ]
5. Have you ever used oral contraceptives (Pills) Yes [ ]No [ ]
6. Do you have immunosuppressive disease like HIV/AIDS?: Yes [ ] No [ ]
7. Do you have a high parity (>4 vaginal deliveries)? Yes [ ] No [ ]
8. Have you ever smoked cigarettes? Yes [ ] No [ ]
9. Have you had sexually transmitted infections/diseases? Yes [ ] No [ ]

10. Is your male partner circumcised? Yes [ ] No [ ]

Thank You.

APPENDIX VII

ETHICAL APPROVAL CERTIFICATE

HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL SHIKA,
ZARIA, NIGERIA.

E- mail: abuthshika@yahoo.com website: www.abuth.org

Chief Medical Director: Prof. Lawal Khalid, MBBS, FMCS, FWACS, FRCS(ED) mni
Chairman, Medical Advisory Committee: Prof Adamu Ahmed: MBBS, (ABU) LLB (ABU), BL, FWACS, FICS, FACS.
Director of Administration: Alh. Abdulraheem Sallau, BA(Pub. Admin) PGDPA, MPA, (ABU) AHAN, ACIPM.
Ag. Chairperson: Prof Aisha I. Mamman MBBS, FMCPath

NHREC No. 10/12/2015
D-U-N-S No. 954524802
ABUTH/HREC/CL/05

DATE: *10th November, 2016*

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Immunocytochemical and Molecular analysis of Liquid-based Cervical Cytology Specimens in Ahmadu Bello University Teaching Hospital Zaria, Northern Nigeria.

ABUTH Ethics Committee assigned number:	-	ABUTHZ/HREC/PO3/2016
Name of the principal Investigator:	-	**Prof. Saad Aliyu Ahmed**
Address of the Principal Investigator:	-	Dept. of Pathology ABUTH, Zaria.
Date of receipt of valid application:	-	26th October, 2016
Date of meeting when final determination On ethical approval was made:	-	7th November, 2016

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and ***given full approval by the Health Research Ethics Committee.***

Please note: this approval dates from **10th November, 2016 - 10th November, 2017**

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

10/11/2016

Prof. Aisha. I. Mamman *MBBS, FMCPath*
Chairperson