THE UTILITY OF P16INK4A EXPRESSION IN UTERINE CERVICAL BIOPSIES WITH/WITHOUT DYSPLASIA AT KENYATTA NATIONAL HOSPITAL

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DECLARATION

I, Dr ZAWADI MUVUNYI, declare that this is my original work, and has not been presented for any award in any other university or institution.

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DEDICATION

To the loving memory of my mother the late Ngave Marie, my father Semukanya Jean and my siblings: Mao, Flori, Mugisha and Dudu.
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LIST OF ABBREVIATIONS

AJCC American Joint Committee on Cancer
ASCUS: Atypical Squamous Cells of Unknown Significance
CIN: Cervical Intraepithelial Neoplasm
CKD: Cyclin/cyclin-dependent Kinase
DNA: Deoxyribonucleic Acid
EDTA: EthyleneDiamineTetraacetic Acid
ERC: Ethics Research Committee
FDA: Food and Drug Administration
H&E: Hematoxylin& Eosin
HIER: Heat Induced Epitope Retrieval
HLA: Human Leukocyte Antigen
HPV: Human Papillomavirus
HR-HPV: High Risk Human Papillomavirus
HSIL: high-gradesquamous intraepithelial lesion
IARC: the International Agency for Research on Cancer
IHC: Immunohistochemistry
KNH: Kenyatta National Hospital
LSIL: low-grade squamous intraepithelial lesion
n: number
NNL: Non-neoplastic lesion
PCR: Polymerase Reaction Chain
PI: principal investigator
RB/pRB: Retinoblastoma gene

SD: standard deviation

SPSS: Statistical Package for the Social Sciences

UON: University of Nairobi
ABSTRACT

Background: Interpretation of intraepithelial neoplasia on hematoxylin & eosin (H&E)-stained cervical tissue sections is subject to substantial rates of discordance among pathologists, resulting in significant impact on patient management. Overexpression of p16INK4a has been shown to be strongly associated with dysplastic and neoplastic epithelium of cervix uteri due to high-risk human papillomaviruses (HR-HPV). Therefore, p16INK4a Immunohistochemistry (IHC) provides valuable additional information in the interpretation of cervical histology with resultant improvement in definitive identification of dysplastic lesions and reduction of inter-observer disagreement in conventional histology.

Objective: To determine the utility of p16INK4a expression in uterine cervical biopsies with or without dysplasia at Kenyatta National Hospital (KNH).

Design: Laboratory-based, retrospective cross-sectional study.

Setting: The University of Nairobi (UoN) Anatomical Pathology laboratories at KNH.

Study population: Colposcopic biopsy specimens reported as negative for dysplasia, CIN1, CIN2 and CIN3 at KNH’s histology laboratory from June 2011 to June 2013.

Main outcome measures: Ninety one previously diagnosed colposcopy biopsies were re-evaluated on H&E and analyzed for p16INK4a expression by immunohistochemistry on paraffin wax embedded tissue blocks. Level of agreement in interpretation of cervical biopsies was compared between primary and review histologic results. Results of p16INK4a expression were correlated with age and histo-morphologic findings.

Results: The age range was between 21 and 65 years (mean 40.2). On primary H&E-stained reports, 37 (40.7%) were CIN2 while 23 (25.3%) were CIN1. On review, 32 (35.2%) were negative for dysplasia and 24 (26.4%) were CIN3. Under half of the cases, 35 (38.5%) showed a
positive p16INK4A expression. The diagnostic test on the previous and review histology exam results indicated a significant difference in the paired outcomes, p<0.001.

There was a significant difference between the review diagnosis and the primary histology results when comparing high grade cases and disease negative cases (p < 0.001), with kappa of agreement of 0.568.

On primary histology results, all (100%) negative for dysplasia and 82% (19/23) of CIN1 cases did not express P16. Almost half (48.6%) of CIN2 were negative for p16INK4a. Majority [80% (12/15)] of CIN3 expressed p16INK4a, p < 0.001.

On review histology result, all (100%) negative for dysplasia and almost all CIN1 (18/19) cases were nonimmunoreactive, p <0.001. There were 11(69%) CIN2 that stained positive for p16INK4a and all CIN3 expressed p16INK4a except one, 23(95.8%) vs. 1 (4.2%).

Conclusion: This study provides strong evidence for the usefulness of p16INK4a immunostaining in improving diagnostic accuracy of cervical biopsy interpretations, by mitigating the significant interobserver variation in interpretation of cervical biopsies on H&E stain. The highest discordance rates were mainly found in CIN1 and CIN2 categories (the “grey area”), while CIN3 showed lower rates of disagreement.

P16INK4a IHC clearly differentiates negative for dysplasia from probable CIN1 on the one hand, and from high grade lesions on the other. This is important because only high grade lesions require further intervention and treatment while negative cases need follow up with screening.

Recommendation: Routine p16INK4a Immunohistochemistry should be conducted for all “grey zone” cases, which include CIN1 and CIN2 in order to inform clinical decisions for surgical intervention or monitoring by screening.
1. INTRODUCTION AND LITERATURE REVIEW

1.1 CERVICAL CANCER EPIDEMIOLOGY AND PREVENTION

Cervical cancer is the third most common cancer in women worldwide, and the seventh most common overall (in both gender combined). In developing countries, cancer of the uterine cervix is ranked second, whereas in developed countries cervical cancer is ranked fifth(1). Globally, cervical cancer accounts for almost half a million new cases annually, nearly one in ten (9%) of all cancers diagnosed in women; with the lowest incidence rates in Western Asia and the highest in Eastern Africa. More than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancers (2).

In many African countries, the true incidence of cervical cancer is not known as there is gross under-reporting and lack of vital cancer registration. Some of the figures quoted in the literature are derived from hospital-based data, constituting only a small fraction of women dying from cervical cancer, as most women lose their lives without receiving any hospital care(3). However, the incidence of cervical cancer has remained high in sub-Saharan Africa; the rate can be up to several-fold greater in poor countries as compared to industrialized ones. This lower rate, in the developed countries, is largely the result of existing rolled out screening tests especially by cytology, and more recently HPV Deoxyribonucleic Acid (DNA) testing for identifying the population at risk in the past 40 years(4). The incidence rates in some countries (Uganda, Mali and Zimbabwe) appear to be on the rise(5)(6).

In Kenya, cervical cancer is the 2nd most frequent cancer among women between 15 and 44 years of age. In 2008, estimates indicate that incidence of cervical cancer was 12.7 per 100,000 women every year, that is to say 2454 women are diagnosed with cervical cancer(7). By age 74,
the cumulative risk of developing cervical cancer was estimated at 2.7%, much greater than the cumulative risk in the world (1.6%).

The incidence of cervical cancer mortality rates varies in different geographical regions. In developed countries where there are successful screening programmes, mortality from cervical cancer seldom exceeds 5 per 100,000 women, while in Africa, especially in East Africa, a mortality rate of 35 per 100,000 has been reported(8). Cervical cancer was responsible for 275,000 deaths in 2008, about 88% of which occurred in developing countries: 53,000 in Africa, 317,000 in Latin America and the Caribbean, and 159,800 in Asia(9). In Kenya, 1,676 women died from the disease in the same year(7).

The survival rate for cervical cancer in sub-Saharan Africa in 2002 was 21% compared with 70% and 66% in the United States and Western Europe, respectively(10). The five-year relative survival rates in Kampala, Uganda and Harare, Zimbabwe in 1990 were 18% and 30% respectively, while in the USA during the same period the rate was 72%(8). In Harare, 77% of 284 registered cervical cancer patients died within three years of follow-up. The overall observed and relative survival at three years were 44.2% and 45.2%, respectively(11).
1.2 HPV IN CERVICAL NEOPLASIA

Human papillomavirus (HPV) is now known to play an important etiological role in the development of cervical cancer(12). About 2000 women, in 22 countries, histologically diagnosed with invasive cervical cancer were investigated with a standard protocol by the International Agency for Research on Cancer (IARC) for the detection of HPV-DNA using Polymerase Reaction Chain (PCR)-based assay, Munoz et al discovered that HPV were present in 99.7%(95-100%) of invasive cervical cancer leading to the conclusion that HPV is a necessary cause of cervical cancer(13). High-risk types such as HPV (16, 18, 45, 31, 33, 45, 52, 58, 35, and 51) are among the most common types found in cervical cancers and are the main factors implicated in cervical carcinogenesis(13,14).

Human papillomavirus types causing cervical cancer vary from one country to another; over 70% of all cervical cancer cases, in any given country, are attributed to only 2 types, HPV16 and HPV 18. About 41–67% of high-gradesquamous intraepithelial lesion (HSIL) and 16–32% of low-grade squamous intraepithelial lesion (LSIL) are also estimated to be HPV-16/18-positive. After HPV-16/18, the six most common HPV types are the same in all world regions, namely 31, 33, 35, 45, 52 and 58; these account for an additional 20% of cervical cancer worldwide(15).

There are four major steps in cervical cancer development: infection of metaplastic epithelium at the cervical transformation zone, viral persistence, progression of persistently infected epithelium to cervical precancer, and invasion through the basement membrane of the epithelium. Infection is extremely common among young women in their first decade of sexual activity. Persistent infections and precancer are established, typically within 5–10 years, from less than 10% of new infections. Invasive cancer arises over many years, even decades, in a minority of women with precancer, with a peak or plateau in risk at about 35–55 years of age.
1.3 SCREENING AND MANAGEMENT OF CERVICAL NEOPLASIA

Fortunately cervical cancer has a long premalignant period that provides the opportunity for screening and treatment before it progresses to cervical cancer(16,(17). The main objective of screening for cancer is to reduce incidence and mortality from the disease. Cervical cancer is the most effectively controlled by screening as compared to other cancers because pre-cancerous lesions can be detected and treated. Detection of cytological abnormalities by microscopic examination of Pap smears, and more recently with HPV DNA testing, and subsequent treatment of women with high-grade abnormalities prevents the development of cancer (18). Many studies have shown that organized population-based cervical cancer screening every three to five years can reduce cervical cancer incidence by 80% (19,20). Women with screen-detected abnormalities, pre-cancerous lesions and early invasive squamous cell cancer on Pap smear need diagnostic confirmation by colposcopy and histology, and further treatment may be required.

Cytology

Screening by cytology (the Pap smear), through well-run screening programmes, is the mainstay of primary screening for cervical cancer. Although the Pap test has never been examined in a randomized controlled trial, consistent observational data supports its effectiveness in reducing both incidence and mortality from cervical cancer(20,21,22,23).

In Iceland, the mortality rate declined by 80% for more than 20 years, and in Finland and Sweden by 50% and 34%, respectively (24). Similar reductions have been observed in large populations in the United States (US) and Canada. Reductions in cervical cancer incidence and mortality were proportional to the intensity of screening. Mortality in the Canadian provinces was reduced most remarkably in British Columbia, which had screening rates two to five times
those of the other provinces. Case-control studies have found that the risk of developing invasive cervical cancer is three to ten times greater in non-screened compared to screened women. Other studies also found that cervical cancer risk increases with long duration following the last normal Pap test or with decreasing frequency of screening. Nonetheless, screening every 2 to 3 years has demonstrated not to increase the risk of finding invasive cervical cancer above the risk expected with annual screening.

In the US, more than a half of all invasive cancers occur in women that have never had a Pap smear; an additional 10 to 20% of cancers occur in women who spent more than five years following the last normal Pap test. Another proportion of one-quarter of US cervical cancers were in women that had an abnormal Pap smear, but did not get appropriate follow-up. In low-resource settings, cervical cancer screening programme using Pap smear test has failed to demonstrate cancer prevention for many reasons: little or no access to screening programme; costs; lack of well-trained personnel causing sampling and interpretation errors, inadequate cytology laboratory infrastructure, and lack of appropriate follow up of abnormal results.

Visual inspection

Various screening approaches like visual inspection after application of acetic acid or Lugol’s iodine which offer a low cost alternative to cytological screening and have the advantage of immediate results, and, if indicated, treatment in one visit; have been shown to significantly reduce cervical cancer mortality in a large randomized controlled trial in resource limited countries.
Colposcopy

The major role of colposcopic practice is the identification of the most abnormal area for biopsy. This diagnostic procedure is performed for women with abnormal smears or with signs or symptoms suggestive of cancer. It uses a speculum and magnification with a built-in light source to visualize the cervix. Application of a dilute acetic acid or Lugol’s iodine results in acetowhiteness of abnormal tissue that will augment the contrast. Directed biopsy can then be performed for any lesions suspected to be high grade or cancerous without an attempt to treat, swab, or scrape the lesion, which might temporarily hinder the pathologist’s reading of the submitted material. Endocervical curettage can be done when glandular disease is suspected or the transformation zone is not visualized in its entirety. Because colposcopic appearance is often complex, and the most abnormal area may be small, the sensitivity of the procedure will depend on taking more than a single biopsy in many cases. Its sensitivity and specificity have been estimated to 54% and 85% respectively. Considering other prevention strategies, colposcopy is being challenged due to its low sensitivity and low specificity, especially among women aged 40 years or older where more than half of all CIN2 are missed (36,37).

HPV testing

The etiologic link between persistent high-risk human papillomavirus (HR HPV) infections and cervical cancer and its immediate precancerous lesions has been widely demonstrated (12). This has led to the use of HPV DNA testing as supportive tool in the cervical cancer screening tests. Detection of high-risk HPV DNA is considered to be potentially useful in three clinical applications: (1) as a primary screening test, solely or in combination with a Pap smear to detect cervical cancer precursors, especially for women aged 30 years and older; (2) as a second (i.e.,
tria) test to select those women with minor cytological lesions or following an equivocal result of Atypical Squamous Cells of Unknown Significance (ASCUS) in a Pap smear who require referral for diagnosis and treatment and (3) as a follow-up test to predict cure or failure of treatment with local ablative or excisional therapy in women treated for high-grade intraepithelial lesions (38,39). From a large randomized trial result, there is evidence about the efficacy of screening with an HPV DNA test to reduce cervical cancer incidence and mortality. This is attributed to its higher long-term negative predictive value (NPV) that permits extending the screening interval without increasing the interval risk of cancer. It has a clinical sensitivity of 90 to 95% for cervical intraepithelial neoplasia grade 2 or 3 (CIN2/3), and a significant reduction of CIN2/3 and cancer among test-negative women in the subsequent screening round. Thus, testing for HPV DNA as a primary screening test has been approved by Food and Drug Administration (FDA) only in conjunction with cervical cytology and in women aged 30 years and older. This is due to its lower specificity (94%) compared with cytology (97%), as HPV DNA testing positive predictive value is low among women younger than 30 years, who have more transient HPV infection that is of little consequence (40,41).

 Screening in vaccinated cohorts

The success of HPV vaccines opens a new era of cervical-cancer prevention. Currently, only prophylactic HPV vaccines have shown promise and are likely to provide important future health gains: reduction in cervical lesions which will lead to a decrease in rates of colposcopy referrals, by almost 40-60% of existing case loads in developed countries, according to the data from vaccination trials. A reduction in background risk by elimination of the most important HPV types would affect cost-effectiveness and timing/intervals of screening programs.
Vaccination will not, however, eliminate screening. Due to the multiplicity of HPV types and the fact that the vaccines are type-specific, continued HPV screening provides the added benefit of HPV surveillance for vaccinated women. Furthermore, not all women will be vaccinated, and the whole generation of women that has already been exposed to HPV type 16 or 18 may not benefit. Thus cervical screening will need to be continued for several more decades (42).

Newer tests (*P16ink4A, HLA Typing, HPV viral load & Physical state, E2/E6 ratio*)

One of the major disadvantages of the current existing screening tests is their inability to discriminate between lesions that will progress and those that do not. To surmount the current tests limitations, a test is required that indicates that an oncogenic HPV virus has already enhanced genetic instability and rendered infected cells susceptible to transformation, that lead to the development of cancer, which is, a test for HR HPV that indicates the virus will exert its oncogenic potential in that particular woman. (42)

*P16INK4A Immunohistochemistry*

A number of studies have focused on the utility of p16INK4a immunostain as a marker of identifying dysplastic lesions caused by HR HPV and also in distinguishing non-HPV-related changes, such as squamous metaplasia, inflammatory conditions or reactive conditions that either mask or mimic dysplasia in cervical biopsy specimen; from HPV-related changes like CIN 1. p16INK4a has recently been described as a surrogate marker for HR-HPV associated squamous and glandular intraepithelial lesions of the cervix. It has been also demonstrated that the immunohistochemical staining pattern of p16INK4a is different in high grade intraepithelial
neoplasia of the cervix (CIN 2 and 3), where it is diffuse, from sporadic or focal staining patterns in CIN 1.

The protein p16INK4a or p16 protein (p16), a CDKN2A gene product, an inhibitor of cyclin-dependent kinases (CDK)-4 and -6, is encoded by the tumor suppressor gene INK4a. The CDKs phosphorylate retinoblastoma protein (pRB), which results in a conformational change and release of E2F from the pRB. The phosphorylation of RB is a molecular "ON-OFF" switch for the cell cycle. P16INK4a inhibits the cyclin-dependant kinases and thereby prevents the phosphorylation of RB, keeping it in the hypophosphorylated form, i.e. its active form. In the hypophosphorylated form, pRB binds to transcription factors responsible for cell cycle progression. In various tumors its control function over a cell passing through G1/S check-point is broken due to mutational or epigenetic events. The RB gene in cervical cancers has been shown to be functionally inactivated as a consequence of HPV E7 protein expression. Owing to the pRB inactivation by the HPV E7 oncoprotein, cells uncontrollably pass to the S- cell cycle stage. In the RB signal pathway p16INK4a acts upstream of pRB. In cells of HPV-positive cervical dysplasia and carcinomas, it loses the role of the G1/S check-point controller. The content of p16INK4a increases drastically as a consequence of pRB inactivation. The feed-back loop between pRB inactivation by the oncoprotein E7 and the sharp growth of the p16INK4a amount had been demonstrated in vitro. Thus, in the p16/cyclin D1/cdk4/pRB cell cycle regulatory cascade, the correlation between pRB and p16 is obvious in various cancers. Various cancers with mutation or deletion of the RB gene show overexpression of p16INK4a, and a reciprocal correlation between pRB and p16INK4a has been established. Experimental studies have demonstrated not only p16INK4aoverexpression in established cervical cancer cell lines but
also marked enhancement of p16INK4a expression in immortalized human ectocervical cells by HPV-16/18(43)(44).

These studies have also suggested that inactivation of the p16/cyclin D1/cdk4/pRB cell cycle regulatory cascade by HPV occurs during the early immortalization step in cervical carcinogenesis, but not during late malignant transformation.

p16INK4a can serve as a biomarker that is independent of the individual HR-HPV type and indicative of the cervical cancer disease process in action. There are several types of HR-HPV but in each case the effect of the E7 oncoproteins is the same in blocking pRB and leading to the over-expression of p16INK4a. This protein is not expressed in the normal cervical epithelium. In Klaes et al study, p16INK4a IHC has been shown to provide additional information in the interpretation of cervical histology. There was significantly better agreement in the interpretation of p16INK4a expression which was restricted to CIN 2/CIN 3, CIN 1 associated with high-risk human papillomavirus, or cervical cancer. p16INK4a immunostaining allowed precise identification of even small CIN or cervical cancer lesions in biopsy sections and helped to reduce interobserver variation in the histopathologic interpretation of cervical biopsy specimens(45)(46).Thus, p16 IHC can reduce false-negative and false-positive biopsy interpretation and thereby significantly improve cervical (pre)-cancer diagnosis,(45) and also p16INK4a negative CIN1 may benefit from a less intensive follow-up as they rarely progress to high grade(47).
2. RATIONALE

The diagnostic interpretation of H&E-stained cervical tissue sections is subject to substantial rates of discordance among pathologists. In order to avoid that, strict criteria for the diagnosis of CIN on cervical biopsy and cone specimens have been proposed (42) such as the presence of koilocytes, the maintenance of an intact basement membrane with a loss of the usual maturation of the squamous epithelial cells at the surface, a 3-fold increase in nuclear size, conspicuous nuclear hyperchromasia with irregular contour of nuclear membranes, among others have been suggested. Despite these criteria, however, discrimination of CIN is still not optimal (48)(49)(50).

As biopsy diagnoses establish the basis for decisions to treat, the impact of an inaccurate diagnosis is significant.

A more specific biomarker, p16INK4a, has been proved to mitigate this lack of inter-observer reproducibility in the histologic diagnosis of CIN. p16INK4a IHC has been shown to provide valuable additional information in the interpretation of cervical histology (46). Over-expression of p16INK4a is a direct marker of the oncogenic activity of all the various high-risk HPV types and has a better agreement when it comes to its interpretation.

This study aims to evaluate the utility and value of immuno-staining for p16INK4a as a diagnostic adjunct in the evaluation of cervical histology and further to contribute to the body of knowledge regarding this new diagnostic test.
3. RESEARCH QUESTION AND OBJECTIVES

3.1 RESEARCH QUESTION

Is p16INK4a immuno-staining useful in improving diagnosis of colposcopic biopsies with or without dysplasia?

3.2 BROAD OBJECTIVE

To determine the utility of p16ink4a immuno-staining applied on colposcopic biopsies with/without dysplasia at KNH.

3.3 SPECIFIC OBJECTIVES

1. To re-evaluate, previously reported H&E stained colposcopic biopsies with or without dysplasia.

2. To determine p16ink4a immuno-staining expression on colposcopic biopsies.

3. To compare H&E morphology diagnosis and the expression of P16ink4a immuno-staining on colposcopic biopsies.

4. To assess the utility of p16INK4a immuno-staining in improving diagnostic accuracy of cervical intraepithelial neoplasia.
4. STUDY DESIGN AND METHODOLOGY

4.1 TYPE OF STUDY

This was laboratory based, retrospective cross-sectional study.

4.2 STUDY AREA DESCRIPTION

Tissue blocks were recruited from KNH histology laboratory and processing of specimens was carried out at the University of Nairobi histology laboratory.

4.3 STUDY POPULATION

Colposcopic biopsies reported as negative for dysplasia, CIN1, CIN2 and CIN3 using paraffin embedded tissue blocks retrieved at KNH’s histology laboratory, from June 2011 to June 2013.

4.4 STUDY ELIGIBILITY CRITERIA

4.4.1 INCLUSION CRITERIA

1. Colposcopic biopsies reported as negative for dysplasia including normal, cervicitis, reactive changes, basal cell hyperplasia, mucosal atrophy and squamous metaplasia.

2. Colposcopic biopsies reported as CIN 1, CIN2 and CIN3

4.4.2 EXCLUSION CRITERIA.

1. Blocks found unsuitable for sectioning due to physical disintegration.

2. Poorly processed tissues.

3. Colposcopic biopsies without representative epithelium for review or sufficient tissue for IHC
4.5 SAMPLE SIZE DETERMINATION

Sample size calculation for proportions (Woolson, 1987)

\[ n = \frac{Z^2 \alpha (P(1 - P))}{d^2} \]

Where

\( Z_{\alpha/2} \) is critical value for 95% confidence interval = 1.96

\( P \) is the estimated prevalence of biopsies at UoN histology lab that are CIN1 and non neoplastic (40%)

\( d \) is the estimated level of precision = 10%

\( n \) is the number of patients required

\[ n = \frac{1.96^2 \times (0.4(1 - 0.4))}{0.1^2} \]

\[ = 92 \]

The final sample size will be calculated by Applying Finite Population Correction (FPC) to N= 728 = Average of 364 colposcopic biopsies per year for 2 years. The minimum estimated number of biopsies \( n' \) within the proposed study period of 2 years required for this study is 81.

\[ n' = \frac{n}{1 + \frac{n}{N}} = \frac{92}{1 + \frac{92}{728}} = 81 Biopsies \]
4.6 SAMPLING METHOD AND CASES IDENTIFICATION

Purposive sampling method was used. The sample collection started following ethical approval. A total number of 152 blocks, corresponding to the diagnostic categories of negative for dysplasia, CIN 1, CIN 2, and CIN 3, were retrospectively retrieved into the study, using the surgical pathology files containing histology reports, going backward from June 2013, as a starting point. The name, age, patients hospital and laboratory number were noted from the histology report as the cases were identified. This information was used to retrieve the archived paraffin embedded blocks.

4.7 MATERIALS AND EQUIPMENTS

Asemi automated Rotary microtome and an Olympus microscope were provided by the department of Human Pathology. p16INK4a immunostaining was done using manual procedure at UoN, histology laboratory, KNH.

4.8 METHODS

4.8.1 PARAFFIN EMBEDDED BLOCK RETRIEVAL

Paraffin wax embedded blocks were retrieved from histology archives of the study site using the laboratory numbers on the surgical pathology reports. Blocks of 127 cases were found, and then transported to UoN histology laboratory where the H&E and IHC staining were done.

4.8.2 HISTOLOGICAL PREPARATION- H&E FOR LIGHT MICROSCOPY

Two to five micron sections were taken from each of the blocks. The sections were mounted on the H&E labeled slides, then stained using the standard H&E staining procedure as shown in
appendix 5. When dry, they were stored serially in a tray according to study numbers. Cases were initially evaluated by the principal investigator, and then independently reviewed by the supervisors. Cases with discordant results were reviewed by a different pathologist who was the tie breaker. On the basis of all readings combined, a “consensus diagnosis” (2 out of 3 in agreement) was reached. This information was then recorded on the data sheet.

For cases with more than 2 tissue blocks, 2 blocks with the highest histologic grade of lesion for each case were included.

4.8.3 SPECIMEN PROCESSING-IMMUNOHISTOCHEMISTRY & INTERPRETATION

Two to 5 micron sections were cut from each of the study blocks and mounted on two Poly-L-lysine treated slides well labeled with lab number and type of test. When dried, they were stored serially in a tray and processed in different batches. Two positive and 2 negative controls were included in each batch and were therefore subjected to the same test conditions as the study cases. The sections were then stained with p16INK4a using manual immunostaining procedure as shown in the appendix 2 and 4.

The evaluation of the p16INK4a stained slides was performed using 3 different staining patterns: negative, focal, and diffuse staining. Negative staining was defined as nonimmunoreactive. Focal staining was defined as noncontinuous staining of isolated cells or small cell clusters. Diffuse staining was defined as a continuous staining of cells. p16INK4a expression was nuclear, as well as cytoplasmic. Only cases with diffuse staining were considered as positive for analytical purposes(51).

All the scores were independently reviewed by the PI and supervisors to achieve a consensus score. This was then scored and entered into the data collection sheet.
4.9 QUALITY ASSURANCE

All reagents were prepared according to the manufacturer’s instructions. Standard operating procedures were adhered to for all procedures. The reagents were checked for expiry date, turbidity, odor and precipitates. The recommended storage conditions for all reagents were observed. Positive and negative controls were included in each batch during IHC staining and interpretation. The slides were well labeled before mounting the section and then arranged in order to avoid mix up of slides. All the scores were independently reviewed by 2 pathologists. Discordant cases were reviewed by a 3rd pathologist as a tiebreaker.

4.10 DATA MANAGEMENT AND STATISTICAL ANALYSIS

4.10.1 DATA COLLECTION AND STORAGE

The age, sex, laboratory, study and patient’s number and p16INK4a immunoreactivity score were entered into the data collection sheets (appendix 1). Histological diagnosis was entered into the data collection sheet after review of morphology on a routine H&E stained section. p16INK4a expression was determined and entered into the data sheet. One slide for H &E and one slide for P16Ink4A expression were processed for each patient.

The data sheets were filed and kept in a secure lockable cabinet. Data sheets were also saved as spreadsheets in soft copies and password protected.
4.10.2 DATA ANALYSIS AND PRESENTATION

Data was entered into SPSS (Statistical Package for Social Sciences) version 15.0 (SPSS Inc), cleaned, verified and password protected. For confidentiality purposes, the personal identifiers in the data were de-identified before importation into the statistical analysis package.

Descriptive statistics used were Means (Standard Deviations) for normally distributed continuous variables such as age. Count and proportions were used for the tabulation of categorical variables.

Bivariate associations were assessed by Pearson chi-squares, T-test and ANOVA (Analysis of variance). The Marginal homogeneity (Stuart-Maxwell) and asymptotic symmetry tests were used to analyze matched-pair data of previous and review histology exam with multiple discrete levels of the outcome variable in place of McNemar which can only handle two level outcome variables only.

For the inter-rater agreement between p16INK4a expressions with H&E findings was measured using the kappa coefficient, and reported using a bootstrapped, bias-corrected method, 95% confidence interval (Reichenheim, 2004; Carpenter et al, 2000) and P-value <0.05 was considered statistically significant.

Data was presented in form of charts including pie charts, histograms and descriptions photomicrographs and tables. All analyses were performed in Stata version 12.1 (StataCorp, College Station, Texas USA).
5. ETHICAL CONSIDERATION

Permission for records and specimen retrieval and use in this study was obtained from the KNH/UON ERC.(Appendix 5)

All patient identifiers were protected and study numbers were used instead of original laboratory numbers to maintain confidentiality.

Any clinically significant discrepancies or additional information that was found in this study were conveyed as an addendum to be used in the continued care of the patient.

The findings were submitted to scientific journals for publication without patient identifiers.
6. RESULTS

A total number of 91 out of 127 cases met the inclusion criteria.

The age range was between 21 and 65 years, with majority being in the age group 31 to 40 years and a mean age of 40.2 years (SD = 10.2). (Figure 1)

![Age distribution](image)

**Figure 1**: Age distribution (n=91)

The most dominant primary H&E-stain reports were CIN2 37(40.7%) and CIN1 23(25.3%). On review, negative for dysplasia 32(35.2%) and CIN3 24(26.4%) were predominant. Under half 35 (38.5%) of the cases had a positive p16INK4A expression.The most dominant primary H&E-stain reports were CIN2. (Figure 2)
Bivariate Analysis of p16 expression and age

The mean age for the cases who either had a positive or negative P16 test result were similar, 39.7 vs. 41.7 years p= 0.666 (Table 1).

Table 1: p16INK4a expression by mean age

<table>
<thead>
<tr>
<th>P16</th>
<th>Mean</th>
<th>SD</th>
<th>Test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-test</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>39.69</td>
<td>10.05</td>
<td></td>
<td>0.666</td>
</tr>
<tr>
<td>Positive</td>
<td>41.66</td>
<td>10.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results of an analysis of variance did not reveal any significant difference in age by previous histology exam result, all p > 0.05 (Table 2).
Table 2: Primary histologic diagnosis by mean age

<table>
<thead>
<tr>
<th>Primary Histology</th>
<th>Mean</th>
<th>SD</th>
<th>Test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>40.25</td>
<td>10.18</td>
<td>ANOVA</td>
<td>0.644</td>
</tr>
<tr>
<td>CINI</td>
<td>41.78</td>
<td>9.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CINII</td>
<td>37.92</td>
<td>10.43</td>
<td>0.231</td>
<td></td>
</tr>
<tr>
<td>CINIII</td>
<td>43.87</td>
<td>10.04</td>
<td>0.155</td>
<td></td>
</tr>
</tbody>
</table>

The age distribution of cases did not vary by the results of review histology, all p > 0.05. (Table 3)

Table 3: Review histologic diagnosis by mean age

<table>
<thead>
<tr>
<th>Review diagnosis</th>
<th>Histology</th>
<th>Mean</th>
<th>SD</th>
<th>Test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ANOVA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40.59</td>
<td>10.26</td>
<td>0.623</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CINI</td>
<td>42.05</td>
<td>9.69</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CINII</td>
<td>38.13</td>
<td>11.71</td>
<td>0.265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CINIII</td>
<td>39.92</td>
<td>9.89</td>
<td>0.502</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On H&E stain, there was a significant difference in the paired outcomes between previous and review histologic diagnosis (Table 4).

Table 4: Difference between primary and Review histologic diagnosis

<table>
<thead>
<tr>
<th>Primary results</th>
<th>CIN1</th>
<th>CIN2</th>
<th>CIN3</th>
<th>NNL</th>
<th>Tests</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>5.26</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIN1</td>
<td>5</td>
<td>68.4</td>
<td>4</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CIN2</td>
<td>13</td>
<td>6.25</td>
<td>12</td>
<td>50</td>
<td>1</td>
<td>3.13</td>
</tr>
<tr>
<td>CIN3</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>50</td>
<td>2</td>
<td>6.25</td>
</tr>
</tbody>
</table>
With dichotomous categorization of the cases in high grade cases including CIN2 and CIN3 on one hand, and disease negative cases (including cases categorized as negative for dysplasia or CIN 1) on the other; there was a significant difference between the review diagnosis and the primary histology results, p < 0.001, with kappa of agreement of 0.568.

Table 5: Difference between primary and review histologic diagnosis

<table>
<thead>
<tr>
<th>Previous result</th>
<th>Low grade</th>
<th>High grade</th>
<th>Tests</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade</td>
<td>68.6</td>
<td></td>
<td>Symmetry (asymptotic)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>35 3 4 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>31.3</td>
<td></td>
<td>Marginal homogeneity</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>16 7 6 90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On primary histology diagnosis, there was a statistically significant difference in the p16INK4a expression, p value < 0.001. All (100%) negative for dysplasia and 82% (19/23) of CIN 1, on primary results, did not express P16INK4a. Almost half (48.6%) of CIN2 were negative for p16INK4a. Only 80% (12/15) of CIN3 expressed p16INK4a. (Table 6)

Table 6: P16 expression versus primary histologic diagnosis

<table>
<thead>
<tr>
<th>Primary diagnosis</th>
<th>Negative n %</th>
<th>Positive n %</th>
<th>test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>16 28.57</td>
<td>0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN1</td>
<td>19 33.93</td>
<td>4 11.43</td>
<td>Pearson Chi square</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CIN2</td>
<td>18 32.14</td>
<td>19 54.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN3</td>
<td>3 5.36</td>
<td>12 34.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
On review histology diagnosis, there was a significant difference in the p16INK4a expression, p value <0.001. All (100%) negative for dysplasia and almost all CIN1 (18/19) cases were nonimmunoreactive. There were 11(69%) CIN2 that stained positive for p16INK4a and all CIN3 expressed p16INK4a except one, 23(95.8%) vs. 1 (4.2%). (Figure 3)

![Figure 3: Comparison of p16INK4a expression and review histologic diagnosis](image)

**Reliability Analysis**

If each histology result was determined randomly, but with probabilities equal to the overall proportions, we would expect the two histology results to agree on 22.96% on morphology. In fact, there was agreement in 47.3% of the samples or 31.5% between random agreement and perfect agreement. The amount of agreement ('fair rating' based on Landis and Koch (1977) interpretation ladder) indicates that we can reject the hypothesis that the determinations are done randomly, bias corrected Kappa (95% CI) = 0.315 (0.214 - 0.450) (52)(53). However the interpretation should be made in consideration of the sample size which is below the minimum of 200.
Figure 4: p16INK4a expression and histologic features of cervical lesions. A: negative for dysplasia (H&E). B shows the lack of expression of p16INK4a in normal cervical epithelia. C and E: high-grade cervical intraepithelial neoplasia lesions on H&E. D and F illustrate p16INK4a expression in high-grade CIN lesions.
7. DISCUSSION

This study evaluated 91 colposcopic biopsies; the age range was between 21 and 65 years, with a mean age of 40.2 years. This is similar to another study by Aslani et al, where patients age ranged from 20 to 80 years with mean age of 39.8 years (54).

Level of agreement of primary and review histologic diagnosis

In this study, there was considerable variation between the histologic assessment of H&E-stained slides for the primary reports and review reports. In interpretation of any spectrum of change, there is usually no problem with interpretation of extreme ends, in this case negative for dysplasia and CIN 3. However, the middle zones (CIN1 and CIN2 – the “grey area” so to speak) would naturally have the highest difficulty in agreement. We found the highest level of agreement was among the negative for dysplasia cases (94%), followed by CIN3 cases with 80%. There was a very low level of agreement in CIN1 cases (22%) and CIN2 had an agreement of only 30%. Majority, almost 61% of the CINI cases were overcalled. This showed that the pathologists in the primary reports had considerable difficulty in distinguishing cervical intraepithelial neoplasia grade I from reactive proliferations of the cervical squamous epithelium including cervicitis, basal cells hyperplasia and viral changes. This may also be attributed to nuclear enlargement in inflammation or squamous metaplasia being confused with increase in NC ratio, overcall of koilocytic changes in edema, etc…

On review, 13 cases (35%) of CIN2 have been downgraded to CIN1. The overcalling cases in previous reports might be attributed to CIN1 cases associated with HPV changes, tangential cut, and inflammation. However this may also be due to the lesion cut through and not present on the review histologic preparation, as it has been shown that the diagnosis on cervical biopsies can vary significantly with levels.
On the other hand, two cases negative for dysplasia were overcalled CIN3. This may be secondary to atypical reactive changes such squamous metaplasia; mucosal atrophy or lesion cut through and not represented on the review histologic slides.

These overcalls cases may simply reflect deficiencies in the diagnostic ability or training of the pathologists, who were however, all active in routine diagnostic histopathology, and had considerable experience. An alternative and more likely explanation may be largely caused by shortcomings in the morphological criteria used to diagnose cervical intraepithelial neoplasia in general.

By categorizing the cases into high grade CIN including CIN2 and CIN3; and disease negative cases (including cases categorized as negative for dysplasia or CIN 1); there were higher rates of agreement in the low grade lesions, almost 90% as opposed to the rates of agreement of only 60.23% in high grade lesions, which is known to show only a low level of reproducibility when being assessed on H&E-stained slides. Various studies have shown significant interobserver variability in histopathologic interpreting cervical biopsy specimens, resulting in substantial number of false positive and false negative results(45).

In Bellina et al study, the overall level of agreement between pathologists was only 50%, with a very low rate of agreement for CIN1 and CIN 2, similar to our study (55).

Other studies (table 7) have also found a significant rate of agreement in interpretation of H&E stained sections.
Table 7: Meta-analysis of rates of agreement in interpretation of H&E stained sections

<table>
<thead>
<tr>
<th>Author</th>
<th>Negative</th>
<th>CIN1</th>
<th>CIN2</th>
<th>CIN3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klaes et al. (2002)</td>
<td>71%</td>
<td>52%</td>
<td>35%</td>
<td>72%</td>
</tr>
<tr>
<td>Bergeron et al. (2010)</td>
<td>76%</td>
<td>42.3%</td>
<td>36%</td>
<td>76.6%</td>
</tr>
<tr>
<td>Galgano et al. (2010)</td>
<td>86.5%</td>
<td>61.9%</td>
<td>47.6%</td>
<td>75%</td>
</tr>
<tr>
<td>Present study</td>
<td>94%</td>
<td>22%</td>
<td>30%</td>
<td>80%</td>
</tr>
</tbody>
</table>

The overall mean $k$ value for the H&E-stain readings was 0.568, indicating an already moderate level of agreement between the previous and review histologic results.

This is more in line with the findings by Bergeron et al (56) who found the level of agreement of 0.566, which was higher than in reports by Stoler et al (46) and Horn (58) with $k$ values of 0.46 and 0.378 respectively.

An earlier study done by Creagh et al (59) found a poor agreement, with $k$ value as low as 0.20. Bergeron and colleagues reported a $k$ coefficient of 0.77.

The results of the histopathologic interpretation of cervical biopsy specimens guide the subsequent management of women who have been screened for cytologic abnormalities and have been referred for colposcopy for further diagnostic workup. On histology, lesions are categorized as high grade, including CIN2 and CIN3; and low grade, including CIN 1. Category of lesions as cervical intraepithelial neoplasia (CIN) grade 2 or 3 (CIN 2 or CIN 3) leads to excisional or ablative therapeutic interventions to remove the abnormal tissue and prevent potential progression to invasive cancerous growth. Therefore, the accuracy in the diagnostic interpretation of cervical biopsy specimens is important to distinguish between low-grade (CIN 1) and high-grade CIN (CIN 2 and CIN 3) to avoid overtreatment of false-positive cases and
undertreatment of false-negative cases. Moreover, this is clinically relevant because on the one hand, many low-grade lesions have the potential to spontaneously regress (60), and, on the other hand, ablational therapy has been shown to have a potentially negative impact on the reproductive outcome of women (61).

**Histologic diagnosis versus p16INK4a expression**

In this study, the degree of p16INK4a expression correlated well with the degree of cervical neoplasia, and this correlation improved considerably with the review results, p< 0.001.

Similar observation was shown in Agoff et al study (51).

In our study, there was no p16INK4a expression in negative and reactive lesions. CIN 1 lesions showed diffuse p16 expression in about 17% and 5%, on previous and review slides respectively. Close to 75% of CIN1 cases which were upgraded to CIN 2 during the review H&E stained slides expressed p16 positivity. This shows the role of p16INK4a in increasing diagnostic accuracy and also as marker of high grade CIN lesions.

P16INK4a positivity in CIN1 lesions was very low compared to the findings of between 50% and 60% in the studies reported by Agoff et al (51) and Klaes et al (46), respectively. Other studies also showed similar findings (Table 8).

<table>
<thead>
<tr>
<th>Author</th>
<th>Negative</th>
<th>CIN1</th>
<th>CIN2</th>
<th>CIN3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aslani et al. (2013)</td>
<td>1.8%</td>
<td>50%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Reuschenbach et al. (2010)</td>
<td>0%</td>
<td>57.1%</td>
<td>73.9%</td>
<td>100%</td>
</tr>
<tr>
<td>Ishakawa et al. (2006)</td>
<td>0%</td>
<td>25%</td>
<td>80%</td>
<td>94%</td>
</tr>
<tr>
<td>Present study</td>
<td>0%</td>
<td>5.3%</td>
<td>69%</td>
<td>96%</td>
</tr>
</tbody>
</table>
A possible reason for this lower expression, in our study, may be due to poor preservation of our formalin fixed, paraffin embedded tissue or may be because of small number of CIN1 cases or majority of CIN1 cases in our studies may be caused by low-risk HPV types. This might also be attributed to high prevalence of HIV-1 and HPV co-infection in our settings, resulting in a decreasing percentage of p16INK4a-positive stained cells in the HIV-positive cervices in both CIN 1 and CIN 2-3, as reported by Nicol et al, 2012(62)(63); however, the basis for this explanation warrants further exploration in our population.

Just above half of previously reported CIN2 cases and 69% of the review CIN2 cases expressed p16INK4a diffusely. p16INK4a was expressed in 80% and almost 96% of CIN3 cases in previous and review reports, respectively, p<0.001.

This explains the role of p16INK4a expression as a maker of high grade lesions. Similar observations were reported by Agoff et al and Bergeron et al(56,51).

8. LIMITATIONS

Limitations of the present study included a relatively small sample size and also its retrospective nature, as some blocks may have been poorly preserved and not showing p16INK4a expression. Proper tissue fixation impacts IHC results and since no control over how this was done in a retrospective study, it may have contributed.

Finally, due to the small nature of punch and forceps biopsies, some cases may have been cut through and levels may have been unrepresentative of clinical lesions or on IHC staining which depends on the nature and level of histologic sections.
9. CONCLUSION

This study provides strong evidence for the usefulness of p16INK4a immunostaining in improving diagnosis accuracy of cervical biopsy interpretations, by mitigating the significant interobserver variation in interpretation of cervical biopsies on H&E stain. CIN1 and CIN2 (the “grey area”) showed the highest discordance in our review, while CIN3 showed lower rates of disagreement.

P16INK4a IHC clearly discriminates between negative and probable CIN1 lesions on the one hand, and high grade lesions on the other. This is important because only high grade lesions require further intervention and treatment while negative cases may be followed up with screening.

10. RECOMMENDATIONS

- Routine p16INK4a Immunohistochemistry should be conducted for all “grey zone” cases, including CIN1 and 2, however it is not meant to replace H&E- stain but, rather as a special or confirmatory stain.

11. CONFLICT OF INTEREST STATEMENT

- The author declares that there is no potential conflict of interest relevant to this study.
12. REFERENCES


64. Roche mtm laboratories AG. Instructions for use of CINtec® Histology kit for the qualitative detection of p16INK4a antigen. REF: 9517. 2006: 12-15

13. APPENDIX

APPENDIX 1 - DATA CAPTURE SHEET.

STUDY TITLE:

THE UTILITY OF P16INK4A EXPRESSION IN UTERINE CERVICAL BIOPSIES REPORTED NEGATIVE AND POSITIVE FOR DYSPLASIA

DATE………………

1. PATIENT IP NUMBER………………

2. LABORATORY NUMBER…………………..

3. STUDY NUMBER…………………………

4. STUDY SITE: THE KENYATTA NATIONAL HOSPITAL

5. AGE (specify in completed years)………………

   20-30
   31-40
   41-50
   51-60
   >60

6. PREVIOUS MORPHOLOGY REPORT

   1   Negative for dysplasia
   2   CIN1
   3   CIN2
   4   CIN3

7. REVIEW OF MORPHOLOGY REPORT

   1   Negative for dysplasia
   2   CIN1
   3   CIN2
   4   CIN3

8. IMMUNO-HISTOCHEMISTRY.

p16^{INK4a} EXPRESSION
p16\textsuperscript{INK4a} EXPRESSION SCORE:

Each lesion was graded according to a 2-tier system: Negative staining, defined as nonimmunoreactive or focal staining; and positive, as diffuse staining both nuclear and cytoplasmic.

APPENDIX 2-P16INK4A IHC PROCEDURE(64)

Manual immuno- staining procedure was performed. After mounting the sections on poly-L–lysine treated slides and loading them into the machine, IHC staining for p16INK4a consisting of a series of the following steps were carried out:

1. Rinse slide twice with Bond wash solution and twice with tris EDTA buffer.

2. Incubate with tris EDTA buffer for 20 minutes at 100 °C.

3. Incubate further with the tris EDTA buffer for another 12 minutes at room temperature. Steps 2&3 are also referred to as heat induced epitope retrieval (HIER). HIER describes a process of heating formalin-fixed paraffin-embedded tissue sections for improved immunoreactivity of tissue antigens with their specific antibodies.

Following antigen retrieval;

4. Rinse three times with bond wash solution.

5. Wash with bond wash solution for three minutes.

6. Block with peroxide for 5 minute

7. Rinse three times using bond wash solution at 35°C.

8. Incubate

10. Apply post primary antibody for 8 minutes and wash with bond wash solution thrice each wash taking 2 minutes.

11. Apply Polymer for 8 minutes and wash with bond wash solution twice each wash taking 2 minutes.

12. Rinse with deionized water.

13. Rinse with mixed DAB refine then incubate the sections with mixed DAB refine for 10 minutes; DAB acts as the chromogen.

14. Rinse with deionized water.

15. Stain with hematoxylin for 5 minutes.

16. Rinse with deionized water,

17. Rinse with bond wash solution,

18. Rinse with deionized water

19. Air-dry.


21. Score P16INK4a expression on the data sheet
APPENDIX 4 -HARRIS HAEMATOXYLIN AND EOSIN STAINING PROCEDURE(65)

**Principle of the stain**

The mordant forms a lake on the tissue. It is on the lake that the stain gets attached thus colouring the cell nuclei. The nuclei having an affinity for the basic radical in the dye retains the colour even after treatment with 1% acid alcohol. Eosin stains the cytoplasm as a counter stain

**Staining technique**

1. Bring section to water
2. Stain in Harris haematoxylin for 5 minutes
3. Rinse in tap water
4. Differentiate in 1% acid alcohol, 3 dips
5. Rinse in tap water
6. Blue in Scotts tap water for 30 seconds or in running tap water for 10 minutes
7. Counter stain in Eosin for 5 minutes
8. Rinse in tap water to remove excess eosin followed by 70% ethanol to obtain the desired shades of red and pink.
9. Dehydrate in the 3 changes of absolute alcohol
10. Clear in 3 changes of Xylene
11. Mount with D.P.X