P16 AND TOP2A IMMUNOHISTOCHEMICAL EXPRESSION FOR DETECTION OF CERVICAL INTRAEPITHELIAL AND EARLY MALIGNANT LESIONS

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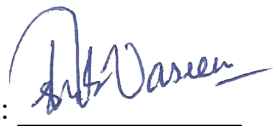
Scholar/ Author's Sign: 

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Dedicated to My Beloved Parents & Husband

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ABSTRACT

Cervical Cancer (CC) is the fourth most common type of cancer worldwide. In vast majority of cases, it is caused by Human Papillomavirus (HPV) - a Sexually Transmitted Disease (STD). In Pakistan, the incidence of cervical cancer is 4.9% while its morbidity and mortality rates are considerably higher due to ignorance in terms of screening, prevention and vaccination. As a result more than 70% women with CC are diagnosed at very advanced stage of malignancy. In this time of vaccines, systematic screening for CC may be considered as one of the most valuable tools for reducing the disease burden and death rates. Despite the characteristic advantages of Liquid Based Cytology (LBC) in cervical screening, LBC has low sensitivity (40-50%) which usually leads to under diagnosis. While HPV testing in cervical screening has been proven effective to some extent but its low specificity may result in unnecessary treatment and high-risk HPV testing alone cannot differentiate between transient and persistent infections. Hence, applying different markers in CC screening is necessary for its control, early-diagnosis and prevention. P16 is a cyclin-dependant kinase inhibitor and plays a crucial role as a cell cycle regulator by decelerating cell progression from G1 to S phase. While Topoisomerase IIA is an enzyme responsible for DNA strands uncoupling during its replication and expressed only in cycling cells. The primary objective of this study was to assess immunohistochemical expression of P16 and TOP2A in normal, pre-cancerous and cancerous cervical cytology cell blocks and to correlate their expression level with cytological and various clinicopathological parameters. It was a cross-sectional study conducted at PNS Shifa Hospital Karachi and Dr. Ruth K. M. Pfau, Civil Hospital, Karachi over a time period of eight months. A total of 60 cervical cytology samples were analyzed for immunohistochemical staining using CDKN2A/P16-INK4a Mouse (IgG) and Topoisomerase II alpha Rabbit (IgG) Monoclonal antibodies. Demographic data and various clinicopathological parameters were recorded using the designed Performa and the results of immunostaining were analyzed and correlated with the documented parameters. The results revealed that there is a significant association of P16 immunohistochemical expression with age group, ethnicity, menopausal status, cytological diagnosis, and cancer status. While TOP2A was only associated with menopausal status, cytological diagnosis, and cancer status of the patients. P16

immunostaining was directly related with the increasing severity of cervical cytological abnormalities. The sensitivity and specificity of P16 for detecting pre-cancerous and cancerous lesion was significantly higher, whereas TOP2A demonstrated higher specificity for detecting cancerous lesions. Therefore, P16 can be a reliable immunohistochemical marker for diagnosing early as well as late cancerous lesion while TOP2A is highly specific marker for ruling out non-cancerous lesion.

KEYWORDS: Cervical cancer screening, Human Papillomavirus, Cervical Intraepithelial Neoplasia, P16, TOP2A, Immunohistochemistry, Liquid-Based Cytology

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LIST OF ABBREVIATIONS

ADC: Cervical Adenocarcinoma

ADSC: Cervical Adeno Squamous Carcinoma

AI: Artificial Intelligence

AJCC: American Joint Committee on Cancer

AMPK: 5' Adenosine Monophosphate-activated Protein Kinase

AP: Alkaline Phosphatase

ASC-H: Atypical Squamous Cells, HSIL cannot be excluded

ASCUS: Atypical Squamous Cell of Undetermined Significance

ASIR : Age-Standardized Incidence Rate

AGCs: Atypical Glandular Cells

BMI: Body Mass Index

CC: Cervical Cancer

CDH1: Epithelial cadherin (E-cadherin)

CDKs: Cyclin Dependent Kinases

CDKI: Cyclin Dependent Kinase Inhibitors

CIN: Cervical Intraepithelial Neoplasia

DAB: Diaminobenzidine

DNA: Deoxyribonucleic acid

EOSI: Early Onset of Sexual Intercourse

FIGO: The International Federation of Gynaecology and Obstetrics

HBV: Hepatitis B virus

HPV: Human Papillomavirus

Hr-HPV: High-risk Human Papillomavirus

HRP: Horseradish Peroxidase

HSIL: High Squamous Intraepithelial Lesion

HSV: Herpes Simplex Virus

IARC: International Agency for Research on Cancer

IHC: Immunohistochemistry

IUD: Intrauterine Device

IL-10: Interleukin-10

IRF-1: Interferon regulatory factor 1

IRF-3: Interferon regulatory factor 3

LBC: Liquid Based Cytology

LKB1: Liver kinase B1

LSIL: Low squamous intraepithelial lesion

MCM2: Minichromosomal Maintenance-2

mTOR: Mechanistic Target Of Rapamycin

NGS: Next Generation Sequencing

NILM: Negative for Intraepithelial Lesion and Malignancy

OCPs: Oral Contraceptives Pills

PAP stain: Papanicolaou stain

RB: Retinoblastoma protein

SCJ: Squamocolumnar Junction

SCC: Squamous Cell Carcinoma

SIL: Squamous Intraepithelial Lesion

STIs: Sexually Transmitted Infections

STD: Sexually Transmitted Disease

TAP1: Transporter associated with Antigen Processing 1

TBS: The Bethesda System

TLR: Toll-like receptors

TNF-α: Tumor Necrosis Factor α

TOP2A: Topoisomerase II Alpha

VIA: Visual Inspection with Acetic acid

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CHAPTER 1

INTRODUCTION

1.1 Background

Cancer is the primary cause of mortality and a major impediment to the raising life expectancy in every country. According to the World Health Organization (WHO) estimation in 2019, cancer is the primary or second most frequent cause of mortality before the age of 70 in 112 out of 183 countries while in another 23 countries it ranks as third or fourth most frequent cause of death among elderly population. Globally, the incidence and mortality due to cancer is increasing at an alarming rate, this can be attributed directly to population growth and ageing along with shifts in the distribution and frequency of the primary risk factors for cancer, many of which are related to socioeconomic development (Sung et al., 2021).

Among the top five malignancies in adult female worldwide, cervical cancer (CC) ranks as the fourth most common type. The rate of cervical cancer incidence is at peak in Eastern African as compared to Western Asia. On the other hand, in South East Asian region it is ranked as second most common cancer among women and the foremost reason of cancer-related deaths in women of low and middle income countries (LMICs). Researches have explained that early onset of sexual activity and rising frequency of human papillomavirus (HPV) infection both influences the increasing incidence of cervical cancer among younger women (Shrestha et al., 2018).

1.1.1 Epidemiology

As shown in Figure 1.1, Cervical cancer (CC) is the fourth most common type of cancer worldwide; in 2022, approximately 661,021 women were diagnosed globally out of which 338,189 expired due to this disease (Bray et al., 2024). In vast majority of cases, cervical cancer is caused by Human Papillomavirus (HPV) - a Sexually Transmitted

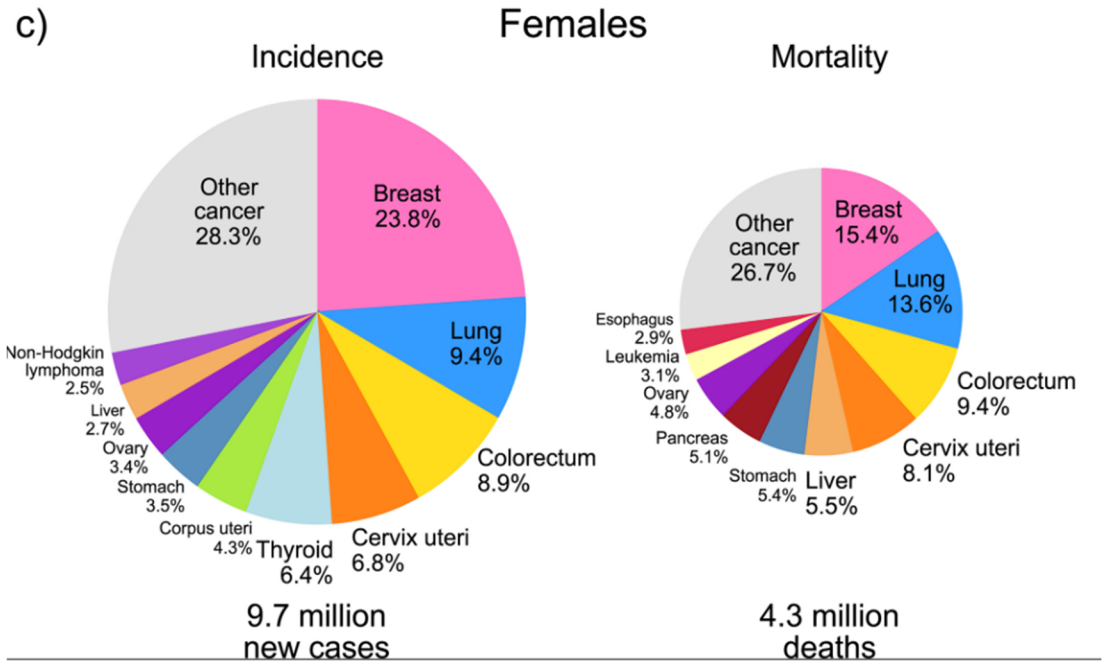
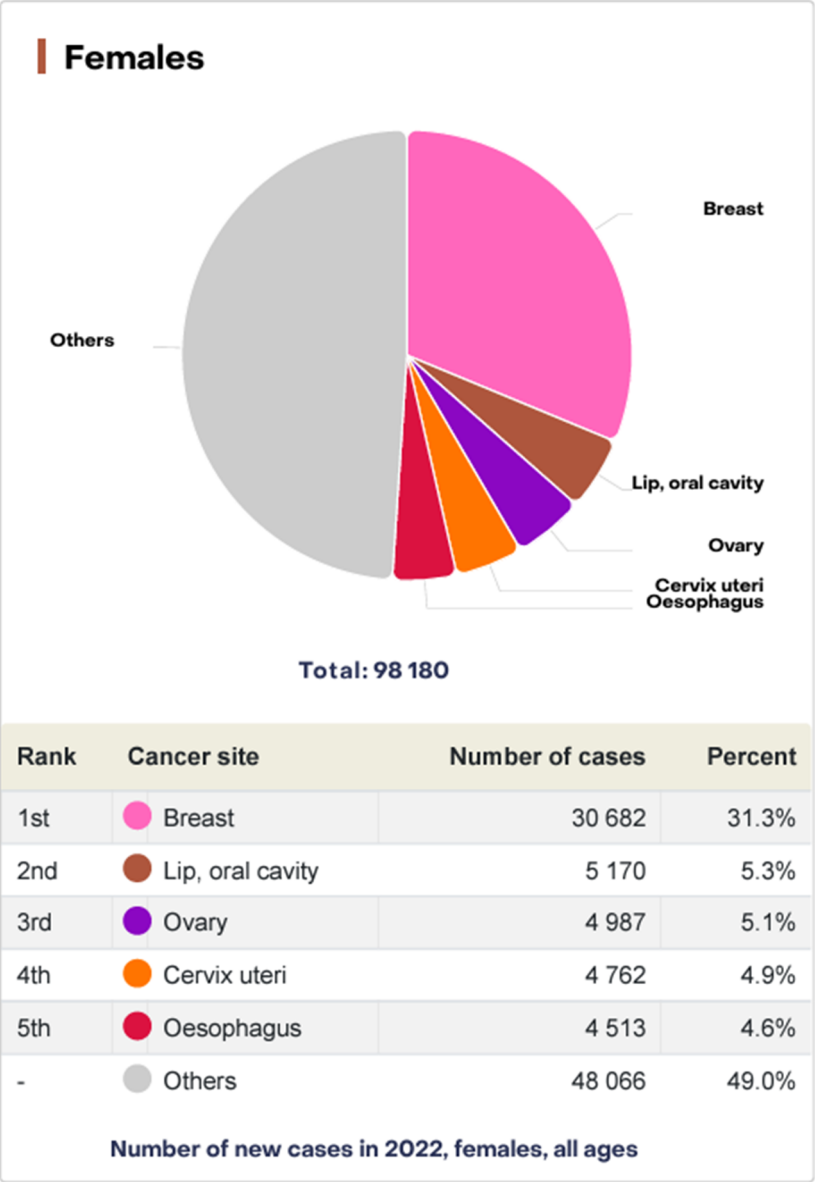


Figure 1.1 Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[1]

Disease (STD); studies conducted in the United States have shown that about 75% of sexually active individuals had been infected corresponding to approximately twenty million active infections, increasing at an annual rate of more than 5 million new HPV cases (Yuan et al., 2021). About 13,000 women in US are diagnosed with cervical cancer, resulting in about 31% fatalities despite screening and treatment (Centers for Disease Control and Prevention, 2022).

As shown in Figure 1.2, the incidence of cervical cancer in Pakistan is 4.9% with mortality rate of 2.6% (Global Cancer Observatory, 2024). According to the recent Shaukat Khanum Memorial Cancer Hospital & Research Centre (2022) collective cancer registry report, cervical cancer ranks as fourth most common cancer with prevalence of 4% in adult females. The national cancer registry of Pakistan (2015-2019) also revealed cervical cancer as the fourth most prevailing malignancy among females with 4.17% prevalence (Ikram et al., 2023). The Karachi Cancer Registry (2017-2021) has also reported cervical cancer in the top ten malignancy among females with the highest age-standardized incidence rate (ASIR) of 11.50. Recent estimations reveal that more than 5000 women in Pakistan are diagnosed with cervical cancer every year out of which

[1] CA A Cancer J Clinicians, Volume: 74, Issue: 3, Pages: 229-263, First published: 04 April 2024, DOI: (10.3322/caac.21834)



Rank	Cancer site	Number of cases	Percent
1st	Breast	30 682	31.3%
2nd	Lip, oral cavity	5 170	5.3%
3rd	Ovary	4 987	5.1%
4th	Cervix uteri	4 762	4.9%
5th	Oesophagus	4 513	4.6%
-	Others	48 066	49.0%

Number of new cases in 2022, females, all ages

Figure 1.2 Pakistan statistics 2022: GLOBOCAN estimates incidence of Top 5 cancer in Pakistan[2]

63.83% women die due to this disease (ICO/IARC HPV Information Centre, 2023). The morbidity and mortality rate from cervical cancer in Pakistan is considerably higher mainly due to the high prevalence of Human Papillomavirus (HPV) infection, insufficient number of feasible and efficacious diagnostic programs, limited awareness campaigns for early detection of the disease, inaccessibility to diagnostic and therapeutic services, patient's compliance, and increasing number of relapses. As a result more than 70%

[2] Global Cancer Observatory. (2024). Statistics at a glance, 2022. In Global Cancer Observatory: Cancer Today [Report]. International Agency for Research on Cancer. https://gco.iarc.who.int/media/globocan/factsheets/populations/586-pakistan-fact-sheet.pdf

women with cervical cancer are diagnosed at very advanced stage of malignancy (Batool et al., 2017).

1.1.2 Risk Factors

Many prior epidemiological studies have been conducted on putative risk factors associated with cervical cancer development and their relationship with the environmental, behavioural, or host immune system, which includes both modifiable and some non-modifiable risk factors.

1.1.2.1 Modifiable Risk Factors

- **Human Papillomavirus Infection**

Human Papillomavirus (HPV), a DNA virus is one of the most potent human carcinogens and has been associated with cancer at multiple sites including cervix. It is the most frequent viral infection of reproductive system and a common sexually transmitted disease worldwide. Usually, HPV infects the mucocutaneous membrane and is responsible for generating viral components within the mature epithelial cells and leads to loss of normal cell-cycle control and is a stimuli for unregulated cell division which results in accretion of genetic damage.

Although there are more than 100 different HPV genotypes, only 14 have been classified as carcinogenic (National Cancer Institute, 2023). In Pakistan high-risk HPV i.e. 16 & 18 are responsible for about 88.1% of invasive cervical cancer (ICO/IARC HPV Information Centre, 2023). More than 70% of females are infected with HPV during their lifespan. Over 90% of newly acquired HPV infections, regardless of age, resolve within 6–18 months, and the emergence of cervical intraepithelial neoplasia (CIN) requires long term persistence of infection. The infectivity period influences the odds of HPV elimination; the rate of clearance decreases with prolong duration of infection. Persistent HPV infections, however, account only for 10% with high-risk types (especially 16 and 18), possibly leading to dysplasias and high-grade cervical cancer in half of these cases (Centers for Disease Control and Prevention, 2022).

Several previous researches have explored the risk factors associated with persistent high-risk HPV infection and the development of cervical cancer. A number of risk factors—such as immunosuppression Stelzle et al. (2021), concurrent sexual infections Yang et al. (2020), risky sexual practices Liu et al. (2015), prolong use of oral contraceptive pills Moscicki et al. (2008) and tobacco use Haverkos et al. (2003)— have been studied in the last three decades.

- **Oral Contraceptive Pills**

Oral contraceptives pills (OCPs) are an extremely potent medications used by women, to avoid unplanned or undesired pregnancies. Globally, around 16% of women use oral contraceptive pills (Bovo et al., 2023). Despite the wide spread usage of oral contraceptive pills, certain unclear risks i.e. development of cervical lesions are need to be considered. Numerous researches have evaluated the link between OCPs use and risk of cervical cancer. The findings, however, are contradictory, ranging from a positive to a negative association and to even an inverse relation. Oral contraceptive pills as a risk factor for cervical cancer is still not a well-established fact.

In the year 2012, International Agency for Research on Cancer (IARC) published data regarding the use of OCPs which shows that its usage may be responsible for an increased risk for some cancers while protective against others. OCPs usage was linked with an increased risk for breast cancer among women less than 35 years of age, in situ and invasive cervical carcinoma, and even liver cancer. Furthermore, the risk for cervical cancer development increases proportionally with the duration of OCPs usage and the risk diminished after its cessation.

The role of oral contraceptives was deemed contentious in a recent study on the risk factor of cervical cancer (Lukac et al., 2018). Another research study concluded that long-term oral contraceptive use was clearly a contributing factor for cervical cancer development, but it could not be proven as the primary cause (‘Cervical Cancer and Hormonal Contraceptives’, 2007). Another study reported that using OC pills significantly increases the risk of cervical cancer (Bond, 2014). Numerous research studies related to OCPs use in women with persistent hr-HPV infection pointed out an

increased risk of high-grade cervical lesions, indicating an increased HPV genomic expression in OCPs users (Bovo et al., 2023). In a univariate analysis, a research study on the usage of oral contraceptives identified risk variables for cervical intra-epithelial neoplasia (CIN); however, the risk was not statistically significant when adjusted for HPV infection (Kjellberg et al., 2000).

In a 10 year follow-up study, the association of CC incidence was observed in OCPs users and non-hormonal intrauterine device (IUD) users. In women with up to 2 years of OCPs use the incidence of pre-malignant lesion was 0.9/1000 women per year while 2.2/1000 women per year for those with around 8 years of OCPs use. However, no difference in the incidence and rate of CC was observed in IUD users (Bovo et al., 2023).

- **Parity**

HPV infection has been established as a crucial risk factor for cervical cancer (Bosch & de Sanjosé, 2007). Nevertheless, this viral infection alone is not responsible for cancer development. HPV in conjunction with other factors leads to progression of infection into cancer (Bosch & de Sanjosé, 2007). Several prior studies have reported a significant association between high parity and cervical cancer (Abacjew-Chmyłk et al., 2016). This increased risk of cervical cancer among women with high parity is due to an association of higher rate of cervical abnormalities during gestation period, higher HPV detection frequency among pregnant females and few studies also suggested that higher vaginal parity is also responsible for local changes in cervical cells due to birth traumas.

In 2002, a multicentric case-control study found that frequency of term pregnancy has a direct linked with risk of squamous cell carcinoma (SCC) development. However, no significant association was found between number of full-term pregnancies and risk of adenocarcinoma or adenosquamous carcinoma (Muñoz et al., 2002). A recent systematic review and meta-analysis of case control studies also concluded that higher parity is positively linked to cervical cancer (Tekalegn et al., 2022). Similar results were also found in local research study, in which parity was a significant risk factor for cervical cancer (Sadia et al., 2022).

- **Smoking**

Cigarette smoking is another major risk factor for cervical lesions advancement (Roura et al., 2014). According to WHO around 0.43 million adults' deaths every year are due to second-hand smoking, 64% of which are females (World Health Organization., 2010). According to the World Bank data, the prevalence of current tobacco use among females in Pakistan was 7% in the year 2020. However, a recent study found that the overall prevalence of exposure to second-hand tobacco smoke was 33.2% among females in Pakistan (Zahra et al., 2022).

A meta-analysis has indicated that in comparison to non-smokers, active smokers have an increased risk for the development of cervical in situ and malignant lesions. Furthermore, the risk increases with the larger number of cigarette smoked per day and persisted among women with positive high-risk HPV infection (International Collaboration of Epidemiological Studies of Cervical Cancer et al., 2006). A prospective study verifies the association between active smoking and cervical cancer and shows that passive smoking is also a risk factor for cervical carcinoma (Trimble et al., 2005). Studies have shown that persistent high-risk HPV infection among women who are long-term tobacco users and heavy smokers are at an increased risk for subsequent high-grade cervical lesions (Fang et al., 2018; Jensen et al., 2012). In another study, pre-cancerous lesions Low squamous intraepithelial lesion (LSIL) and Atypical Squamous Cell of Undetermined Significance (ASC-US) were independently related with early first sexual encounter and smokeless tobacco use (Shin et al., 2019).

Active smoking women have a 14-folds more risk of cervical cancer and about 24-folds greater risk for developing an advanced cervical lesions (Zidi et al., 2020). The association between increased risks for CC with tobacco smoking has been proven in a number of studies since early 21st century (Haverkos et al., 2003; Moore et al., 2001). This association can be related to the genotoxic effect of smoking to the cervical epithelium, its effect on HPV infected cells through local immunosuppression or malignant transformation (Gunnell et al., 2006).

Second-hand smoke or passive tobacco smoking is also thought to be an important risk factor for cervical carcinogenesis, but the results have been contradictory in related studies. A study by Su et al. (2018) reported 1.7- times increased risk for CC among women exposed to second-hand smoking, while another studies stated that in the absence of active tobacco smoking, passive smoking is not a standalone risk factor for invasive cervical lesions (An et al., 2018). A similar study concluded that in the absence of active tobacco smoking, second-hand smoking is linked with the risk of CIN 1 (Min et al., 2018). These contradicting results might be due to the difference of the smoking pattern or characteristics including number of cigarette smoke daily and duration of smoking among patients with cervical cancer. However, the convincing relation between cervical carcinogenesis and passive tobacco smoking has not yet been evaluated.

- **Concurrent Sexual Infections**

Sexually transmitted infections (STIs) correspond to a serious public health concern globally, particularly among childbearing age women (Arefaynie et al., 2020). On average, globally more than 1 million people acquire an STI daily, mainly trichomoniasis (156 million cases), chlamydia (127 million cases), gonorrhea (87 million cases), syphilis (6.3 million cases) and infections caused by Herpes Simplex Virus (HSV), Hepatitis B virus (HBV), Human Immunodeficiency Virus (HIV), and Human Papilloma Virus (HPV) although majority of these remain asymptomatic (WHO, 2023). A positive correlation between cancer and STIs has also been discovered. Women who reported three or more sexual partners in the previous 12 months had the highest likelihood of HPV infection. HPV infection was the primary risk factor for cervical abnormalities, with a history of STIs, except Chlamydia trachomatis, increasing risk to a lesser extent. Despite the fact that behavioural factors can increase risk, HPV infection affects all sexually active women (Roset Bahmanyar et al., 2012).

Evidence from prior studies suggest that people demonstrating high-risk behaviours i.e. recurrent tobacco and alcohol use, drug abuse, engaging in unsafe sexual practices or having multiple sexual partners are at greater risk of acquiring STIs (Rodríguez-Álvarez et al., 2018; Son et al., 2016; T. L. Wu et al., 2020; Wendland et al., 2018). Other risk factors contributing to STIs includes exposure to physical and sexual

violence, lower literacy rate, low socioeconomic status and early onset of sexual activity (Nigatu et al., 2020).

A number of prior studies have suggested an association of STIs with cervical lesion. A study reported that abnormal cervical cytology and cervical dysplasia was observed in HIV-positive cases in correlation with HPV (Bisherwal et al., 2016). In another study, the association between trichomonas vaginitis and high-risk HPV 16 infection was investigated and the study concluded that co-infection with HPV 16 and trichomonas vaginitis is an important factor for the detection of cervical lesions (Yang et al., 2020). A similar study explored the co-infection with Chlamydia trachomatis and high-risk HPV (particularly 16, 18, 31, 33, 53, and 56 genotypes) and the results showed that it is the most significant risk factor for the incidence of cervical cancer, specifically in younger females, women with early sexual activity, women having multiple sexual partners and oral contraceptives users (Mangieri et al., 2023; Suehiro et al., 2021). A latest study also showed positive association of STIs history with pre-cancerous lesion detection (Abera et al., 2023).

- **Obesity**

The prevalence of obesity is increasing globally. Several studies have demonstrated a direct correlation between obesity (BMI >30) and overweight (BMI >25) with the risk of developing a number of malignancies. This correlation can be attributed to the changes in endogenous hormone metabolism, inflammatory response related to obesity, synthesis of certain cytokines and proteins in addition to genetic factors (Gu, 2013). Obesity is a risk factor for higher cancer related deaths including cervical cancer. Moderate to severely obese women as compared to women with normal BMI have greater than 3-folds increased risk of death from cervical malignancy (Kim et al., 2021).

Although cervical cancer is generally a preventable disease, the association of obesity and high mortality rate due to cervical cancer is alarming. In developed countries, the incidence of CC has been effectively decreasing by routine Pap smear screening tests and HPV vaccination. A study by Urbute et al. (2022) indicates that obese women are less probable to report prompt CC screening despite at greater cancer risk. Therefore,

delayed diagnosis, poor prognosis and more early CC related deaths observed among obese women which may be explained by decrease adherence rate to the screening recommendations (Gnade et al., 2020; Gu, 2013; Sand et al., 2023)

The correlation of obesity with cervical carcinogenesis is still a debatable issue. Contradictory results have been found in many epidemiological studies. A study reported no significant difference in the likelihood to report sexual behavioral risk factors among obese women that may rise the probability of HPV infection and CC (Wee et al., 2008). Another study by Taye et al. (2021) revealed that high BMI (>25kg/m2) is a protective factor of cervical pre-cancerous lesions while in a recent study no association was found between obesity and cervical intraepithelial neoplasia (CIN) (Ssedyabane et al., 2023). However, decrease frequency of pre-cancerous lesions and greater incidence of CC detection were correlated with higher than normal BMI in the latest cohort study (Urbute et al., 2024).

- **Younger age at first Coitus or Marriage at younger age**

The term Early Onset of Sexual Intercourse (EOSI) is described as having a first sexual encounter before 15 years of age. The factors linked to EOSI includes drug abuse disorder (Peltzer & Pengpid, 2015; Roman Lay et al., 2021; Turi et al., 2020), lack of family planning education (Turi et al., 2020), a childhood abuse history (Z.-Y. Wang et al., 2019) and insufficient family support (Gazendam et al., 2020). Early initiation of sexual activity along with engaging in risky sexual practices among young females predispose them to unplanned pregnancies, abortions, STIs and financial strains (Jung, 2019; Nigatu et al., 2020). The mean age of sexual activity initiation is unidentified worldwide.

Early onset of sexual activity has been proposed as a potential risk factor for STIs and progression of cervical lesions to cancer. A number of studies have examined the relationship between early onset of sexual activity and CC. A study compared the people of early sexual activity initiators with late initiators and concluded that individual with premature sexual initiation are more prone to the risk of STIs, accidental pregnancies and HIV infections (Roik et al., 2018). Another study observed that among women of

childbearing age with no history of multiple sexual partners EOSI was associated with STIs particularly in urban areas (Perez-Fernandez et al., 2023). Additional researches highlighted the association of cervical cytological abnormalities with the age of first coitus (An et al., 2018; Umakanthan et al., 2023). Prior study also noted that early marriage and early gestation or childbirth significantly increases the risk of CC. Subsequently, visual inspection with acetic acid (VIA) screening for cervical dysplasia was positively associated with early marriage and young age at first coitus (Ogunbowale & Lawoyin, 2008). A randomized controlled trial reported that short interval between menarche and the onset of sexual intercourse is a significant risk factor for cervical cytological abnormalities and high-grade lesions (Ruiz et al., 2012). However, in a subsequent clinical trial contradictory results were found, short interval between menarche and the onset of sexual intercourse does not elevate the probability of HPV-related cytological atypia (Adhikari et al., 2019).

- **Multiple Sexual partners**

Multiple Sexual partners is a known risk factor for HPV infection which is a key reason behind cervical cancer development. However, its independent role in cervical carcinogenesis is still not evident. Consistently several studies have shown that risky sexual behaviour is the principal cause of HPV infection, particularly in individuals with multiple sexual partners (Yamaguchi et al., 2021).

Early sexual initiation and having multiple sexual partner was significantly associated with childhood maltreatment (Z.-Y. Wang et al., 2019). A study reported that high probability of multiple sexual partners was frequently correlated with early sexual initiation (T. L. Wu et al., 2020). A number of studies have proven that multiple sexual partners were frequently observed in males as compared to females (Ma et al., 2009; Urassa et al., 2008; Wendland et al., 2018). Another study reported that individuals with multiple sexual partners in conjunction with those who did not practice routine barrier contraception methods are at an increased risk of STIs which has an association with cervical neoplasia (Jung, 2019). A recent retrospective study showed that Low squamous intraepithelial lesion (LSIL) was the most common cytological abnormality followed by high squamous intraepithelial lesion (HSIL) and atypical cell of undetermined

significance (ASCUS) (32.6%, 28.8% and 27.4%) in individuals with multiple sexual partners (Umakanthan et al., 2023). Therefore, based on these studies the association between number of sexual partners of an individual and cervical cancer incidence remains even after adjusting for HPV infection. Hence, this suggests that women with multiple sexual partners are the high-risk population for cervical cancer development.

- **Low socio-economic status**

Low socio-economic status of women in developing countries pose a significant risk for cervical cancer, considering the limited access to the high quality healthcare facility. As discussed previously cervical cancer is a preventable disease having a favourable prognosis if identified at an initial stage. Consequently, women in lower-income countries frequently have higher CC morbidity and mortality rates due to less healthcare and prompt diagnostic facilities (Chayo et al., 2023).

Numerous researches have been conducted to find association between low socioeconomic status and CC. A study reported that lower socioeconomic status was strongly associated with early sexual activity among females (Gazendam et al., 2020). A recent study concluded that monthly salary is one of the major cofactor for HPV infection and development of cervical lesions (Mangieri et al., 2023). A systemic review by Chayo et al. (2023); Donkers, Bekkers, et al. (2021) reported that Low socioeconomic status seems to be linked with poor survival rate in patients with CC however, a contradictory results were found in another retrospective study which concludes that there is no significant associations between socioeconomic status and cancer mortality or cancer recurrence in CC patients (Donkers, McGrane, et al., 2021). A similar study showed that women with high socioeconomic status and post-secondary education had high probability of being screened for CC than women with no education and lower socioeconomic status (Dutta et al., 2018). In another study by Sadia et al. (2022) education level was found to be a risk factor for CC. The likelihood of suspicious lesion among women with primary education was comparatively 68% lower than women with no education (Abera et al., 2023). A similar study showed that level of education i.e. college degree or higher is a protective factor of cervical pre-cancerous lesions (Taye et al., 2021).

1.1.2.2 Non-Modifiable Risk Factors

- **Age**

Age is the most important non-modifiable risk factor of cervical cancer. The age-specific incidence rate (ASIR) of CC increases after the age of 25 years. In high-income countries the maximum age limit of CC incidence was around 40 years, while in low-income countries the incidence rate of CC continued to rise significantly up to 55-69 years. The mean age at CC diagnosis was 53 years worldwide however, the average age at death was 59 years. The global peak incidence of CC is 50-54 years although a greater set of countries had maximal incidence of CC among ≥85 years of age group (Arbyn et al., 2020).

Many previous studies have reported that detection of advanced stage CC in elderly population exhibits poor prognosis related to the delayed screening and diagnosis (Gultekin et al., 2018; Wright et al., 2012). Similar results were observed in developed countries where elderly population is susceptible for an increased risk of advanced CC, poor prognosis and more recurrence rate (Bhatla et al., 2019). Another study in developed country suggests that the cervical cytological screening among elderly women should not be discontinued because of a greater risk for an invasive lesions and poor prognosis (Birge et al., 2022).

- **Ethnicity**

Ethnicity plays a pivotal role in the development of cervical cancer. Highest incidence rate of cervical squamous cell carcinoma (SCC) was observed in Black women followed by Hispanic women while the highest incidence rate of cervical adenocarcinoma (ADC) was observed among White women followed by Hispanic women predominantly for localized ADC. However, the highest incidence of cervical Adenosquamous carcinoma (ADSC) was observed among Hispanic women. Regardless of CC subtype and stage, Black women exhibited the highest overall mortality rates and lowest 5-year survival compared to the other ethnic groups especially with regional and distant ADC (Cohen et al., 2023).

- **Family History**

Family history is an important risk factor of cervical cancer particularly Squamous Cell Carcinoma (SCC). A cohort study showed that the risk of cervical SCC was 74-80% higher among women with a positive history of cervical SCC in the first-degree relatives (i.e. mother, sister, and daughter) as compared to the general population. Similarly, the risk of cervical adenocarcinoma was 39-69% greater among women with a positive family history in the first-degree relative (Hussain et al., 2008). These finding presumably suggests that both environmental factors i.e. HPV infection along with some genetic factors are responsible for cervical cancer development.

- **Genetic factors**

The role of genetics in causing CC is still relatively unexplored, although prior studies show evidence that these cases occur within families. A review published in 2004 assessed the association of family history with the probability of cervical in situ or invasive lesions (De M. Zelmanowicz & Hildesheim, 2004). The majority of the studies reported one to two-folds increased risk for CC among individuals with positive history in first-degree relative. However, strong evidence of genetic role in CC development was reported in late 20th century where an increased risk was observed in the first-degree relative i.e. biological mother and sisters as compared to non-biological relatives (P. K. Magnusson et al., 2000).

Previous genetic studies also suggest that certain genetic factors are responsible for persistent HPV infection and progression of precancerous lesion into CC by various pathways. Single Nucleotide Polymorphism (SNP) has been linked to persistent HPV infection and advanced cervical lesions. SNPs inhibit several important gene functions i.e. DNA repair mechanisms, altering immune function, viral infection and cellular entry (S. S. Wang et al., 2009, 2010). Some studies also reported the association of certain Human Leukocyte Antigen (HLA) and haplotypes with an increased risk of CIN3 or CC (Kamiza et al., 2020).

1.1.3 Pathological Basis of Cervical Cancer

The cervix is a dynamic, cylindrical-shaped and fibromuscular segment of female reproductive system which connect the uterine cavity with the vagina externally. It is approximately 4 centimetres long and 3 centimetres wide structure. The cervical canal consists of two types of lining epithelium i.e. endocervix which is lined by glandular-type epithelium (a single layer of columnar epithelial cells), and ectocervix which is lined by multiple layers of squamous epithelium, with flat cells on top. These two type of epithelium overlap at the squamocolumnar junction (SCJ) (Robbin, 2020).

HPV is a small, non-capsulated, circular DNA virus belonging to the Papillomaviridae family with a diameter of about 52–55 mm. The circular genome is a double-stranded DNA molecule of about 8000 base pairs that contains the genetic material that codes for the viral proteins. This genome is encased in an exterior protein shell known as capsid, which contains 72 capsomeres. The HPV genome has about 8 open reading frames (ORFs) all are transcribed from a single DNA strand. These ORFs are divided into 3 functional regions:

- The Early region (E): Encodes proteins involved in viral replication, transcription, and transformation of host cells.

- The Late region (L): Encodes structural proteins necessary for virion assembly. The capsid has two primary structural proteins, i.e., L1 and L2.

- The Long control region (LCR): It is a non-coding region that contains cis elements necessary for viral DNA replication, transcription and regulates the expression of viral genes.

These viral E proteins are transcribed from the early promoter, while the L proteins are transcribed principally from the late promoter (International Agency for Research on Cancer, 2007).

Human papillomavirus infection especially oncogenic subtypes are the main causative agent of cervical neoplasia and targets the immature squamous epithelial cells of the transformation zone as shown in Figure 1.3. Most of these infections are transient and resolve within few months by eliciting an acute and chronic inflammatory response. However, a subset of HPV infection remains and progresses to precursor lesion from where majority of the invasive tumor arises. HPV detection is clearly evident in nearly all the cases of pre-cancerous and cancerous lesions.

Even though HPV infects the immature squamous epithelial cells of the basal layer but the viral DNA replication occurs in the differentiated squamous epithelial cells. Usually, matured squamous epithelial cells do not replicate DNA, however, HPV-infected squamous epithelial cells replicate viral DNA as a result of expression of two important viral oncoproteins i.e. E6 and E7. These two oncoproteins play a crucial role in inactivation of two key tumor suppressors, RB and P53 and stimulate uncontrolled cell growth and increase susceptibility to further mutations that ultimately lead to cervical carcinogenesis. In more than 70% of the cases, oncogenic strains of HPV particularly 16 and 18 are contributory factor for CIN and carcinoma. These oncogenic types have the tendency to integrate into the host cell genome, which is associated with the progression of cervical lesions (Robbin, 2020).

Although, there is an association between persistent HPV infection and cervical carcinoma, HPV infection alone is not sufficient to propel the neoplastic process. There are several other factors i.e. immunosuppression, concurrent sexual infections, risky sexual behaviour, prolonged use of oral contraceptive pills and tobacco use result in the progression of precursor lesion into cancer.

The Bethesda System (TBS) is a standardized reporting model for cervical cytology specimens as shown in Figure 1.4 and 1.5. In addition to its consistent results, it also displays present understanding of cervical cancer. The recent Bethesda System (TBS) has replaced 3 stages of Cervical Intraepithelial Neoplasia (CIN) with only 2 stages i.e. Low-grade Squamous Intraepithelial Lesions (LSIL) and High-grade Squamous Intraepithelial Lesions (HSIL) which could be used to categorize any squamous abnormality.

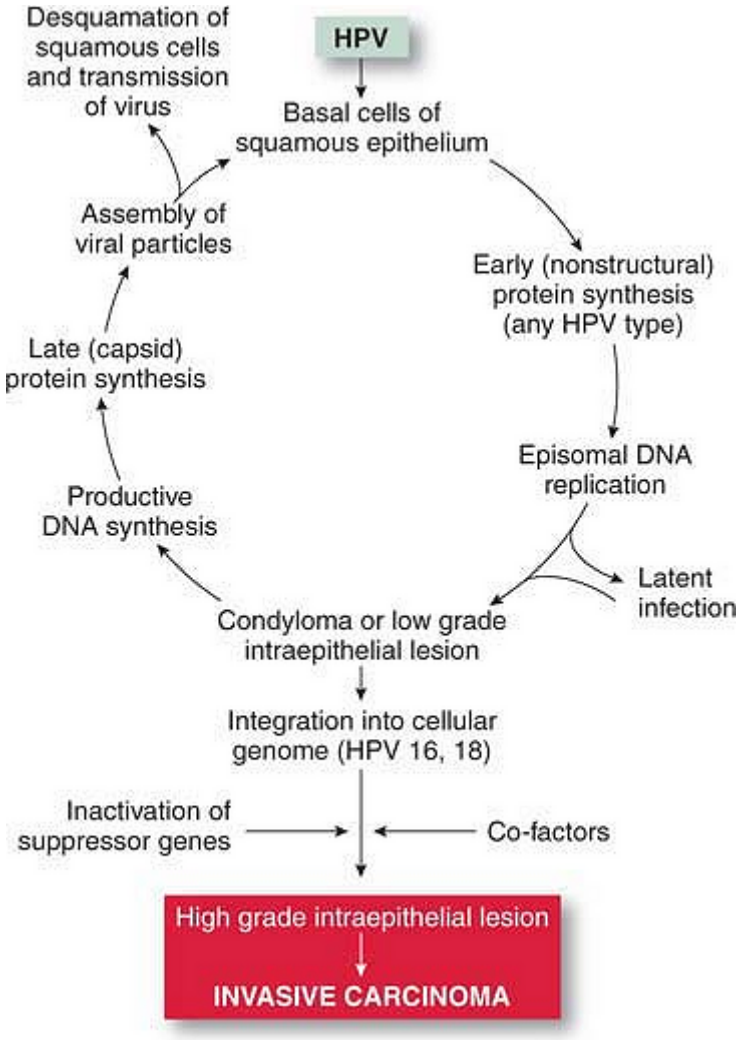


Figure 1.3 Role of human papillomavirus (HPV) in the pathogenesis of cervical neoplasia[3]

The cervical precancerous lesions are primarily epithelial cell abnormalities comprising of Squamous Intraepithelial Lesion (SIL), which incorporates a variety of squamous lesions from Low-grade Squamous Intraepithelial Lesions (LSIL) to High-grade Squamous Intraepithelial Lesions (HSIL) and eventually to invasive carcinoma as shown in Figure 1.6. Although, based on the limitation of the specimen, some ambiguous morphological features which are suggestive of squamous cell abnormalities may come under the grey zone area of Atypical Squamous Cells (ASCs) which are further classified into 2 classes Atypical Squamous Cells of Undetermined Significance (ASC-US) or Atypical Squamous Cells, HSIL cannot be excluded (ASC-H) respectively.

[3] Rubin's Pathology Clinicopathologic Foundations of Medicine Seventh Edition

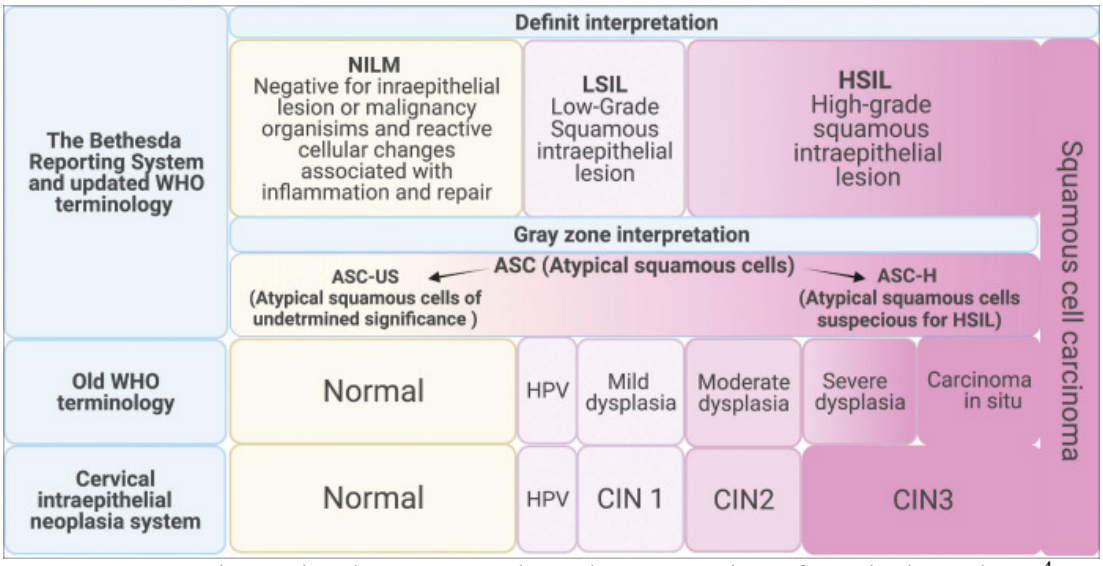


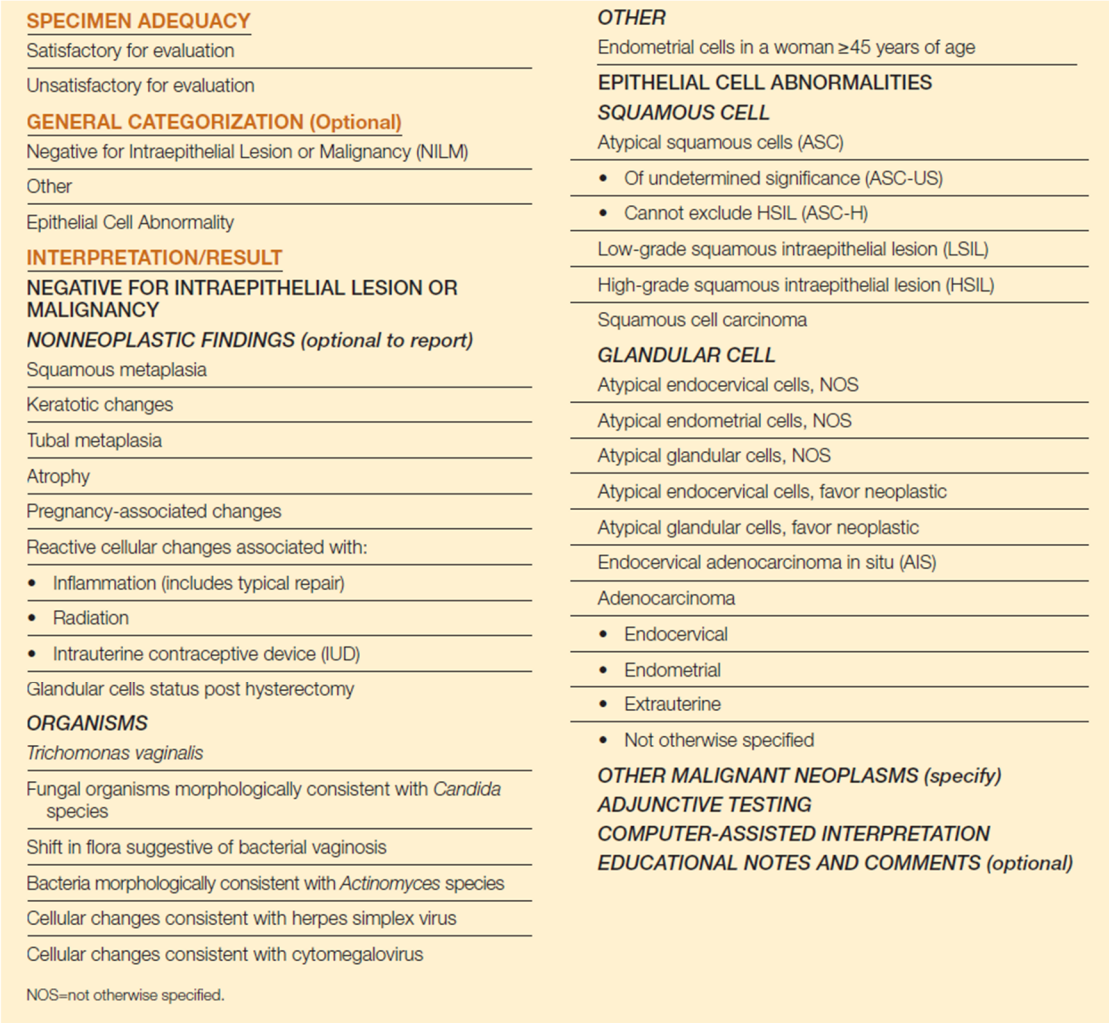
Figure 1.4 The Bethesda system and cytology reporting of cervical specimen[4]

1.1.4 Molecular Basis of Cervical Cancer

HPV targets epithelial cells of the basal layer lying on the basement membrane and completes its life cycle through the epithelial differentiation pathway. HPV infects the basal epithelial cells, likely by micro-abrasions on the surface epithelium. HPV enters into the cell through lateral extension of basal cells that occur during wound healing. Viral internalization continues several hours, after that viral DNA is liberated from the capsid and transferred as a free genetic material into the nucleus.

Early gene expression in basal epithelial cells is strictly regulated, with abundant viral DNA amplification. Replication takes place exclusively in supra-basal i.e. differentiating cells, which are destined for maturity and senescence and consequently do not exhibit the replicative machinery critical for virus survival. To overcome this issue, HPV encodes two essential oncoproteins i.e. E6 and E7 which promote cell proliferation, extending cell cycle progression while inhibiting the programmed cell-death (apoptosis). Thus, cells become accommodating for viral particles and numerous copies of HPV are formed within a cell. L1 and L2 are the viral capsid protein that are expressed in the most superficial epithelial layers, where viral assembly begins and lastly newly formed virions

[4] Alrajjal A, Pansare V, Choudhury MSR, Khan MYA, Shidham VB. Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of Uterine Cervix and Bethesda System. Cytojournal. 2021 Jul 17;18:16. doi: 10.25259/Cytojournal_24_2021. PMID: 34345247; PMCID: PMC8326095.



SPECIMEN ADEQUACY
Satisfactory for evaluation
Unsatisfactory for evaluation

GENERAL CATEGORIZATION (Optional)
Negative for Intraepithelial Lesion or Malignancy (NILM)
Other
Epithelial Cell Abnormality

INTERPRETATION/RESULT

NEGATIVE FOR INTRAEPITHELIAL LESION OR MALIGNANCY

NONNEOPLASTIC FINDINGS (optional to report)
Squamous metaplasia
Keratotic changes
Tubal metaplasia
Atrophy
Pregnancy-associated changes
Reactive cellular changes associated with:
- Inflammation (includes typical repair)
- Radiation
- Intrauterine contraceptive device (IUD)

Glandular cells status post hysterectomy

ORGANISMS
Trichomonas vaginalis
Fungal organisms morphologically consistent with *Candida* species
Shift in flora suggestive of bacterial vaginosis
Bacteria morphologically consistent with *Actinomyces* species
Cellular changes consistent with herpes simplex virus
Cellular changes consistent with cytomegalovirus

NOS=not otherwise specified.

OTHER
Endometrial cells in a woman ≥45 years of age

EPITHELIAL CELL ABNORMALITIES

SQUAMOUS CELL
Atypical squamous cells (ASC)
- Of undetermined significance (ASC-US)
- Cannot exclude HSIL (ASC-H)

Low-grade squamous intraepithelial lesion (LSIL)
High-grade squamous intraepithelial lesion (HSIL)
Squamous cell carcinoma

GLANDULAR CELL
Atypical endocervical cells, NOS
Atypical endometrial cells, NOS
Atypical glandular cells, NOS
Atypical endocervical cells, favor neoplastic
Atypical glandular cells, favor neoplastic
Endocervical adenocarcinoma in situ (AIS)
Adenocarcinoma
- Endocervical
- Endometrial
- Extrauterine
- Not otherwise specified

OTHER MALIGNANT NEOPLASMS (specify)
ADJUNCTIVE TESTING
COMPUTER-ASSISTED INTERPRETATION
EDUCATIONAL NOTES AND COMMENTS (optional)

Figure 1.5 The 2014 Bethesda System for Reporting Cervical Cytology[5]

shed from the surface epithelium which causes new infection. It takes around 2-3 weeks for HPV to complete the lifecycle i.e. the time required for a cervical cell to migrate from basal layer to the most superficial layers, where they mature and undergo senescence.

After HPV infection, viral DNA replicates from episomal DNA and early HPV gene i.e. E1, E2, E3, E4, E5, E6 and E7 are expressed. Further replication of viral genome in the superficial layers results in the expression of late genes L1, L2 and E4. The progression of cervical precancerous lesion into microinvasive or invasive cancer is linked with the viral genome integration into the host chromosomes, also correlated with loss of E2 and consequently E6 and E7 oncogene upregulation.

[5] Cytology Diagnostic Principles and Clinical Correlates, Fifth Edition, Edmund S. Cibas, MD.

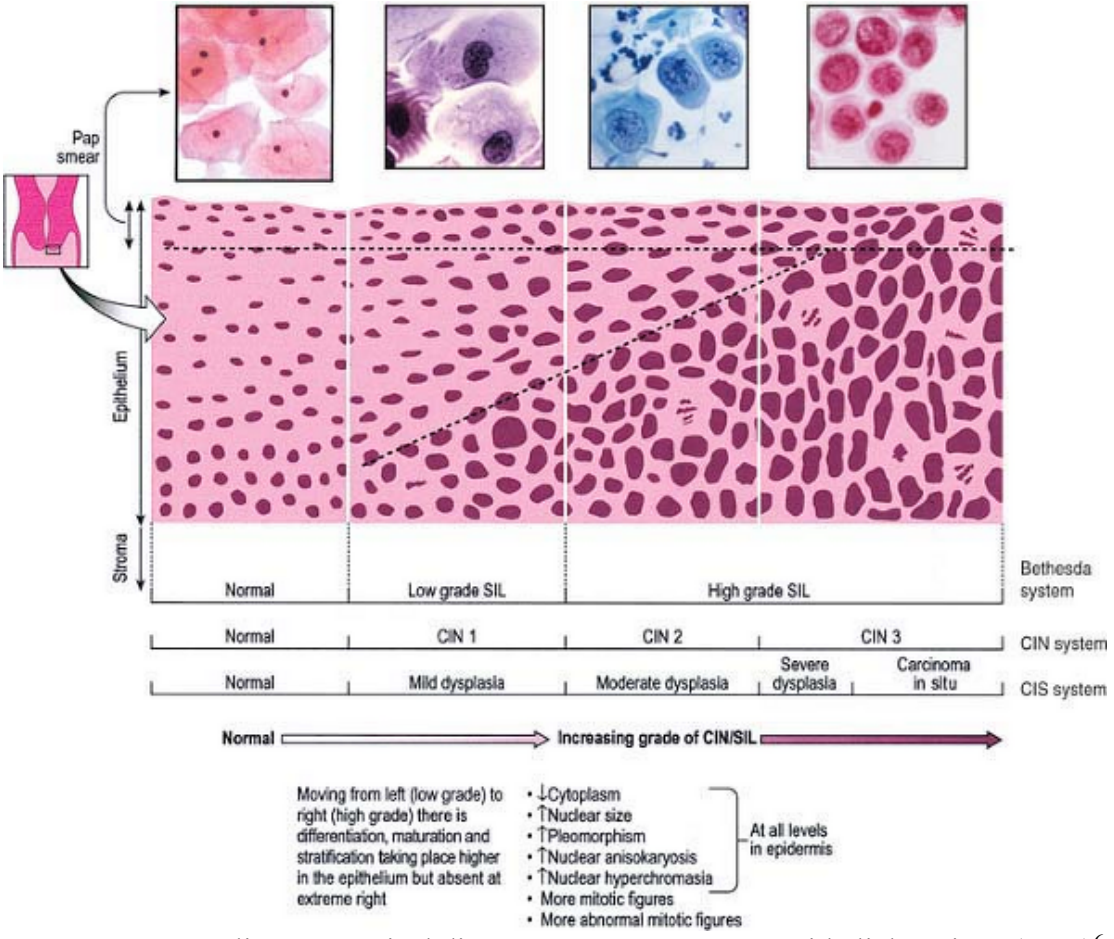


Figure 1.6 Premalignant cervical disease as Squamous Intraepithelial Lesions (SILs)[6]

1.1.4.1 Malignant Transformation

The completion of viral infectious lifespan is a mandatory prerequisite for virion assembly and release, terminal differentiation of the cell is essential for it. Nevertheless, in some high-risk HPV infections, the E6 and E7 oncoproteins are efficient at blocking negative cell cycle regulators that stops the infected cell to achieve maturity. The infected cell continues to actively progress through the phases of cell cycle and cease programmed cell death (apoptosis). This genomic instability will eventually lead to accumulation of genetic alterations, ultimately leading to malignant transformation of HPV infected cells.

The primary oncoproteins in high-risk human papillomavirus (HPV) strains, E6 and E7 target important cellular proteins— pRB (Retinoblastoma protein) and p53 as

[6] Rubin's Pathology Clinicopathologic Foundations of Medicine Seventh Edition

shown in Figure 1.7. p53 has a fundamental role in guarding the genomic integrity by forcefully inducing programmed cell death or arrest cell cycle until errors within the DNA replication can be repaired. The E6 oncoprotein of high-risk HPV causes degradation of p53 tumor suppressor via ubiquitin pathway, thereby preventing apoptosis and allowing replication of transformed cells (Kim et al., 2019).

In contrast, the E7 oncoprotein of high-risk HPV acts as the main transforming protein by inhibiting pRB and disrupting its role in regulating cell-cycle arrest. E7 binds to the retinoblastoma family pocket proteins i.e. RB1, RBL1, and RBL2 and leads to their degradation. This results in release and activation of E2F transcription factors, which triggers the cell-cycle S-phase genes expression, particularly those encoding cyclin A and E, eventually, causing cell-cycle entry and promoting DNA synthesis (Kim et al., 2019).

1.1.4.2 Immune Evasion

Cancer development depends not only on the effective negative regulation of cell-cycle control, but also on the complex immune evasion approaches that allow the virus to remain undetected for extended periods of time. Viral antigens can only be detected in superficial epithelial cells that are bound to be desquamated and escape immunological monitoring. High-risk HPV strains have evolved many strategies to reduce their probability to be detected by the immune system.

E6 oncoprotein of high-risk strains decreases the CDH1 surface expression on epithelial cells, thus limiting their capability to present HPV antigens. Antigen presenting cells (APC) are activated by the Toll-like receptors as an innate immune response to the viral infection. E6 and E7 oncoproteins expression by high-risk strains block toll-like receptor-9 transcription. E7 oncoprotein also inhibits the activation of certain cytotoxic T-lymphocytes by downregulating the expression of TAP1, an important component of the peptide processing and presentation pathway. Furthermore, E6 and E7 oncoproteins block interferon production via certain interactions with IRF-1 and IRF-3. The high-risk HPV lowers the expression level of proinflammatory cytokines, i.e. tumor necrosis factor α (TNF-α), while upregulating anti-inflammatory cytokines i.e. Interleukin-10 (IL-10), which prevent immune cells from migrating to the infection site.

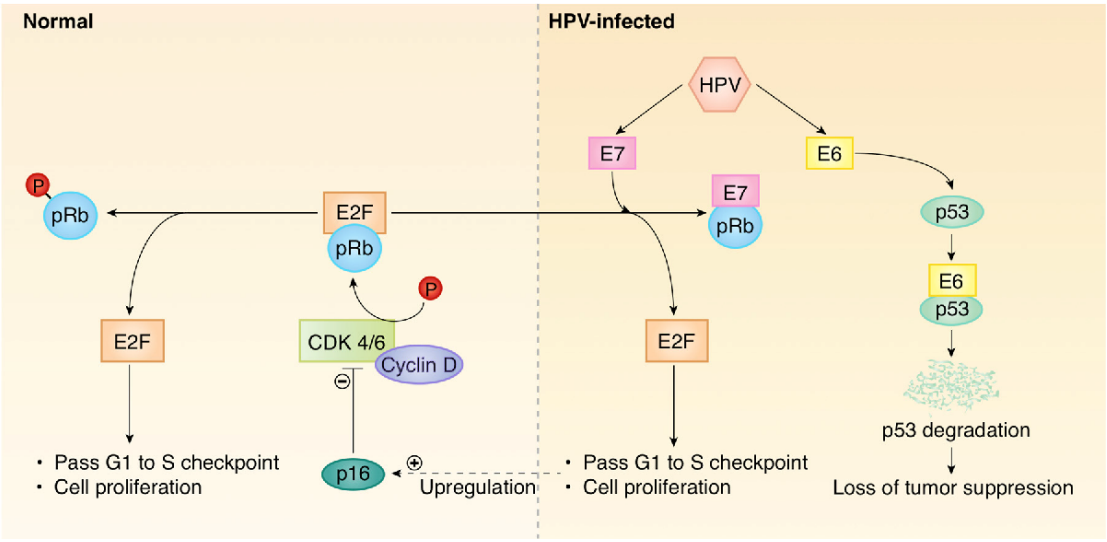


Figure 1.7 Left panel: Normal cell cycle regulation. Right panel: HPV infection disrupts cell cycle regulation through p53 and pRb inactivation, leading to increased P16 expression.[7]

Preinvasive lesion that leads to cancer formation acquires genetic mutations which assists in immune evasion. This process, referred to as cancer immunoediting, is the outcome of the immune system's constant pressure on the developing tumor. The tumor may exhibit MHC class I downregulation and decreased antigen-processing capabilities, as well as escaping T-cell-mediated death, enhanced immunosuppressive cytokine production and immunosuppressive T-regulatory cell infiltration.

1.1.5 FIGO Staging

Invasive CC spreads through direct extension into the parametrium, uterus, vagina, and surrounding organs, i.e. the urinary bladder and rectum. It also extend to the lymphatic channels through regional pelvic lymph nodes. Distant metastasis occurs later to liver, lungs, and skeleton via the hematogenous pathway. In 1958, FIGO assigned the first clinical stage classification for cervical cancer. Following FIGO staging, Pathologic (TNM) staging was then used to document the nodal and metastatic disease status. As shown in Figure 1.8, the FIGO Gynaecologic Oncology Committee revised the staging classification to allow for the allocation of the stage based on three factors i.e. clinical, radiological, or pathological evidence as available. These revision of classification were

[7] Wai, K. C., Strohl, M. P., Van Zante, A., & Ha, P. K. (2020). Molecular Diagnostics in Human Papillomavirus-Related Head and Neck Squamous Cell Carcinoma. Cells, 9(2), 500. https://doi.org/10.3390/cells9020500

made to improve overall patient survival and disease management more precisely. Treatment options vary depending on the stage of disease including both fertility-sparing and non-fertility-sparing surgical procedures, and for locally advanced disease chemoradiotherapy is better option.

1.1.6 Immunohistochemistry and Cervical Cancer

Immunohistochemistry (IHC) is a widely use technique to detect specific proteins within the cells. The principle behind this technique is to use antibodies which bind specifically to antigens within the cells or tissue. This antigen-antibody complex can be visualized via colour precipitates catalyzed by enzymes i.e. Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP). Localization and distribution of specific cellular components within the cells can be detected with the help of IHC. Various IHC markers are been utilized for early detection of cervical precancerous and cancerous lesions.

1.1.6.1 P16 and Cervical Cancer

P16INK4a, also referred to as P16, is a protein that functions as an inhibitor of Cyclin Dependent Kinases (CDKs), playing a crucial role in regulating the cell cycle. Its primary function is to inhibit the phosphorylation of the Retinoblastoma protein (RB) and act as a regulator of cell cycle progression. In normal cells, the expression of P16 is under the negative control of the RB1 gene product, resulting in low levels of P16. However, in HPV-associated tumors, particularly those influenced by the E7 oncoprotein of high-risk HPV, the functional inactivation of RB leads to a substantial increase in P16 expression (McLaughlin-Drubin et al., 2013). Consequently, overexpression of P16 can be studied as a marker of virus-induced cell cycle dysregulation, infection with HPV and increased expression of viral oncogenes. At present, P16 immunostaining has been conducted on resected specimens or cervical biopsy to increase the diagnostic performance of Cervical Intraepithelial Neoplasia (CIN) as an adjunctive diagnostic method (Goyal et al., 2020).

1.1.6.2 TOP2A and Cervical Cancer

The TOP2A gene encodes an important nucleic enzyme known as Topoisomerase II Alpha (TOP2A). This enzyme is responsible for unwinding supercoiled DNA strands

Stage	Description
I	The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded)
IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion ≤5 mm[a]
IA1	Measured stromal invasion ≤3 mm in depth
IA2	Measured stromal invasion >3 and ≤5 mm in depth
IB	Invasive carcinoma with measured deepest invasion >5 mm (greater than Stage IA); lesion limited to the cervix uteri with size measured by maximum tumor diameter[b]
IB1	Invasive carcinoma >5 mm depth of stromal invasion and ≤2 cm in greatest dimension
IB2	Invasive carcinoma >2 and ≤4 cm in greatest dimension
IB3	Invasive carcinoma >4 cm in greatest dimension
II	The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
IIA	Involvement limited to the upper two-thirds of the vagina without parametrial involvement
IIA1	Invasive carcinoma ≤4 cm in greatest dimension
IIA2	Invasive carcinoma >4 cm in greatest dimension
IIB	With parametrial involvement but not up to the pelvic wall
III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes
IIIA	The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause)
IIIC	Involvement of pelvic and/or para-aortic lymph nodes (including micrometastases)[c], irrespective of tumor size and extent (with r and p notations)[d]
IIIC1	Pelvic lymph node metastasis only
IIIC2	Para-aortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV
IVA	Spread of the growth to adjacent pelvic organs
IVB	Spread to distant organs

Figure 1.8 FIGO System for Staging of Cervical Carcinoma[8]

specifically during the S-phase of the cell cycle. Its primary functions are to facilitate DNA replication and ensure proper chromosome separation during cell division, contributing to an accurate transmission of genetic information (Goyal et al., 2020). TOP2A is found in high levels in rapidly dividing cells, such as cancer cells, and is often used as a biomarker for certain types of cancer. Studies have demonstrated a positive association between the overexpression of TOP2A and the transition from Cervical Intraepithelial Neoplasia grade 2 (CIN 2) to more advanced cervical lesions (Sung et al., 2021).

1.1.7 Clinical Features of Cervical Lesions

Usually, cervical pre-cancerous and cancerous lesions are asymptomatic in the early stages. But, the most prominent clinical symptom of CC is abnormal vaginal

[8] N. Bhatla et al., 'Revised FIGO staging for carcinoma of the cervix uteri', Int. J. Gynaecol. Obstet. Off. Organ Int. Fed. Gynaecol. Obstet., vol. 145, no. 1, pp. 129–135, Apr. 2019, doi: 10.1002/ijgo.12749.

bleeding i.e. intermenstrual bleeding, post-coital bleeding or post-menopausal bleeding. Other clinical features include blood-stained or foul smelling vaginal discharge, dyspareunia, lower abdominal pain or heaviness and history of weight loss. However in advanced stages, patient can present with the symptoms of rectal bleeding, loin pain, haematuria, and/or radiculopathy as a consequence of invasive tumor into the adjacent structures.

1.2 Research Gap/Rationale of the Study

Multiple studies conducted mainly in developed countries have shown promising results in the use of P16 and TOP2A cellular biomarkers for early detection and prognosis of cervical cancer. However, limited research to explore their advantages in early diagnosis of disease in facilities with high cervical cancer burden and resource constraints areas remains critical. The main objective of this study is, therefore, to analyze the efficacy of these markers in the context of Pakistan and hence provide a sufficiently sensitive and specific diagnostic tool for cervical cancer. It is expected that the outcome of the study will also be applicable to other low income countries of the world where cervical cancer is highly prevalent.

1.2.1 Theoretical Gap

There are several cellular biomarkers used in the early detection of cervical cancer that have been investigated in various studies. Nonetheless, a limited number of studies have researched the most promising biomarkers for early detection of the disease. This study aims to fill this gap.

1.2.2 Contextual Gap

Limited research has been conducted in Pakistan regarding the early detection of cervical cancer, and so far, very few studies have explored the combined utilization of P16 and TOP2A as early biomarker. No study conducted in Pakistan has yet uncovered the significant biomarkers for the early detection of cervical cancer lesions.

1.2.3 Methodological Gap

Several studies have been conducted worldwide on the application of P16 and TOP2A using immunohistochemistry (IHC) in various contexts, including cervical cancer screening. Yet, only very limited datasets are available globally with regards to immunohistochemical screening of cervical cytology specimen (cell blocks), and even more critical is the nonexistence of such studies specifically in the case of Pakistan.

1.3 Statement of the Problem

Cervical cancer accounts for significant morbidity and mortality among women living in limited resource countries, such as Pakistan. Although the early diagnosis of this disease through cytological screening (pap smear) has improved the outcome for cervical cancer patients, there exists substantial interobserver variation which might lead to misdiagnosis. It is expected that the use of new suitable molecular and cellular markers could potentially improve overall treatment and prognosis. P16 and TOP2A are being investigated worldwide as potential biomarkers for the early detection of cervical cancer. Numerous research studies have demonstrated that the expression patterns of P16 and TOP2A can effectively differentiate between normal cervical tissue, pre-cancerous lesions, and early-stage cervical cancer. These biomarkers show promise in providing valuable diagnostic information and aiding in the early identification of cervical cancer. However, while these studies are promising, more research is needed to fully evaluate the potential role of P16 and TOP2A as an early diagnostic biomarker for cervical cancer.

1.4 Research Questions and Hypothesis

1.4.1 Research Questions

1. Is there a significance of P16 and TOP2A expression occurring in cervical cytology specimens for early diagnosis of pre-cancerous and cervical cancer lesions?

2. Is there an association between P16 and TOP2A expression with the cytological and clinicopathological parameters of cervical cancer cases?

1.4.2 Hypothesis

- **Null Hypothesis**

  - There is no significant overexpression of P16 and TOP2A in cervical cytology specimens of pre-cancerous and cancerous lesions

  - There is no association of P16 and TOP2A expression with clinicopathological parameters of cervical cancer

- **Alternate Hypothesis:**

  - There is significant overexpression of P16 and TOP2A in cervical cytology specimens of pre-cancerous and cancerous lesions

  - There is an association of P16 and TOP2A expression with clinicopathological parameters of cervical cancer

1.5 Objective of Study

- To assess immunohistochemical expression of P16 and TOP2A in normal, precancerous and cancerous cervical cytology cell block

- To correlate P16 and TOP2A expression level with cytological and clinicopathological parameters

1.6 Significance of Study

The specificity of cytologic screening is undoubtedly high, but in developing areas like Pakistan where there is a lack of infrastructure and trained cytologists, it is usually difficult to perform high quality cytologic testing. Besides, Liquid Based Cytology (LBC) is not sensitive enough to detect early cervical lesions compared to molecular markers. Detection of high-risk HPV as a primary cervical screening modality is superior to LBC,

but it is less specific as high-risk HPV testing alone cannot distinguish between transient and persistent infections. Therefore, utilizing different markers in CC screening is necessary for its control, early-diagnosis and prevention in our community.

The significance of P16 in CC screening lies in its ability to differentiate between benign (transient infection) and pre-cancerous or cancerous lesions. Whereas, TOP2A expression determines the proliferative activity of cervical lesions. Heightened levels of TOP2A have been consistently observed in both high-grade CIN and invasive cervical cancer. It is expected that assessing the expression levels of both in cervical cytology specimens will aid in risk stratification, guiding appropriate management, and distinguish between benign and potentially malignant lesions. Moreover, incorporating these immunostaining analysis into CC screening protocols will improve the accuracy and reliability of detecting pre-cancerous and cancerous lesions, leading to earlier intervention and improved patient outcomes.

CHAPTER 2

LITERATURE REVIEW

Cervical Cancer is the fourth most common cancer type among women worldwide with 6.5% incidence and a significant mortality rate of about 7.7% (Sung et al., 2021). High-risk HPV infection is an established causative agent, detected in more than 99% of the cervical pre-malignant and cancer cases. Most of these infection are transient and clear spontaneously yet persistent infection leads to cervical intraepithelial or adenocarcinoma in situ lesions. The transition from dysplasia into invasive carcinoma takes a long period of time in most women. However, in about 10% of the women this transition takes less than a year. Numerous studies have highlighted the association of various factors such as high parity, cigarette smoking, early onset of sexual activity, unhygienic conditions, co-infection with other sexually transmitted infections i.e. HSV and HIV, long-term oral contraceptive pills use with an increased likelihood of persistent HPV infections. Two high-risk HPV serotypes 16 and 18 are responsible for more than 70% of CC cases. Nevertheless, unlike other types of cancers, this disease is practically preventable through various primary and secondary measures, such as HPV vaccination and screening.

2.1 Preventive Measures for Cervical Cancer

HPV is a highly preventable infection; limiting HPV spread is the first line of defence against cervical cancer. This infection can be prevented by refraining from early sexual activity, practicing mutual monogamy, or using barrier contraceptive methods—although this strategy is not 100% effective. Nevertheless, the primary prevention of CC lies within the effective HPV vaccination programs. The introduction of first bivalent and quadrivalent HPV vaccines in 2006 has demonstrated over 90% efficiency in preventing high-risk HPV 16 and 18 infections, which are related to high-grade cervical dysplasia (Crosbie et al., 2013). In 2018, a nine-valent HPV vaccine was shown to be effective in 16 to 26 years old young women for up to six years. About 90% global instances of

cervical cancer may be avoided and extensive coverage could be possible with nine-valent HPV vaccine (Huh et al., 2017).

For some malignancies, including cervical cancer, screening is a crucial secondary preventive method. The basic idea of cancer screening is to identify the disease in asymptomatic, seemingly healthy individuals at an early, treatable stage. The cervix is readily evident due to its anatomic accessibility, thus collecting samples from it is generally easy and painless. Pap smear cytology was the first and is currently the most used screening test for cervical cancer. The test entails staining the cells taken from the transformation zone of cervix with Pap stain and examining them under a microscope.

2.2 Best Screening Modality for Cervical Cancer Past, Present and Future

Various studies have been conducted lately to identify better screening tools for early diagnosis of cervical cancer. After the introduction of conventional Papanicolaou smear (short Pap smear) screening technique, the mortality rate due to CC has decreased by about 70% (Singh et al., 2012), even though the sensitivity of Pap smear ranges from 5-60% with maximum sensitivity reaches up to 80% (Strander et al., 2007). Conversely, this technique is associated with increased incidence of false-negative results in about 20-50% of the cases (Strander et al., 2007). Therefore in the late 20th century, Liquid-Based Cytology (LBC) technique was introduced in order to overcome this issue and to show promising results in reducing false-negative results and significantly improves the sample quality and lower inadequacy rate (Akamatsu et al., 2012; Treacy et al., 2009).

Despite its characteristic advantages in cervical cancer screening, LBC also has low sensitivity (40-50%) which usually leads to under diagnosis (M.-Z. Wu et al., 2019). A study by Ronco et al., (2014) reported that while HPV testing in cervical cancer screening has been proven safe and effective to some extent with greater sensitivity, negative predictive value and optimum reproductivity of the results, however its low specificity may result in unnecessary treatment, irrelevant colposcopy referrals, cause anxiety for women involved and increase overall management costs (Wentzensen, 2013). In recent studies, HPV testing in conjunction with liquid-based cytology test or utilization of biomarkers has proven to increase both sensitivity as well as specificity of HPV testing

in detecting cervical intraepithelial grade 2 or more lesions, and has exceptional benefits clinically as a screening modality. Moreover, the increasing application of approved vaccines may pose challenges for cervical cancer screening due to limited HPV genotype coverage, leading to a significant concern of adapting the current screening methods to accommodate vaccinated individuals (Sundström & Elfström, 2020).

2.3 Challenges and Disparities in Implementing Preventive Strategies

The implementation of the aforementioned preventive strategies, nevertheless, has remained uneven across countries. In the year 2020, more than 80% of HPV vaccination programs had been implemented in high-income countries, whereas only less than 30% such initiatives were reported in the majority of underdeveloped countries (Sung et al., 2021). As a result, low and middle income countries bear a much greater burden of cervical cancer cases than high-income countries, which can be attributed to the stark differences in financial and technical resourcefulness and capabilities. The availability of trained personnel and techniques to implement appropriate treatments has, therefore, significantly decreased the incidence of cervical cancer in more developed areas, but many developing countries are far less privileged where such programs are limited or even non-existent.

Recognizing the substantial global burden of cervical cancer cases and the growing disparities among nations, the Director General of the World Health Organization (WHO), in 2018, issued a call for global action to restrict the disease incidence up to 4 cases per 100,000 women. This ambitious goal is proposed to be achieved through three intervention strategies. Firstly, it is aimed to vaccinate against HPV 90% of girls by the age of 15 years. Secondly, the target is to have 70% women between the ages of 35 and 45 years undergo screening at least twice. Lastly, the strategy includes the treatment of about 90% of all the identified pre-cancerous lesions detected during screening (Canfell et al., 2020). Even then, this plan has been predicted to produce over 74 million cases along with 62 million deaths till the next century (Canfell et al., 2020).

2.4 Importance of Early Detection of Cervical Precancerous Lesions

The overall relative five-year survival rate from cervical cancer is 67.4% and if identified at localized stage (I and II) the relative five-year survival rate is up to 91.1% indicating the significance of early detection in lowering the overall mortality rate due to cervical cancer (National Cancer Institute, 2021). Therefore, routine screening of cervical cancer via Pap smear and /or HPV DNA testing plays a pivotal role in early detection of the cases (National Cancer Institute, 2021). Different strategies especially in developing areas must be implemented to ensure timely and consistent access to the screening.

Prior investigations have showed that Pap smear or HPV DNA testing alone has low sensitivity and specificity. Besides, there is a significant inter and intra observer bias while interpreting the cytological diagnosis between different pathologists which might lead to misdiagnosis and/or underdiagnosis. A study by Palma et al. (2009) reported that accuracy and reliability of the morphological interpretation by pathologist for CIN2 lesion diagnosis is below 50%, while for CIN3 lesion diagnosis the accuracy and reliability is about 81-84% respectively (Carreon et al., 2007). There are significant discrepancies in cytological diagnosis for CIN2 lesion and consequently there are more chances of false-positive and false-negative results. Therefore, recent literature suggests that using immunohistochemical staining could enhance the accuracy of CIN2 lesion diagnosis and ultimately aids in the early detection of cervical pre-cancerous lesion and could potentially improve the overall prognosis of these patients.

Evidently, the early detection and treatment of pre-cancerous lesions is expected to prevent malignancy and hence minimize the escalation of cervical cancer cases. In the past few years, many researchers and scientists have investigated diagnostic pointers in the early detection of cervical cancer comprising of HPV DNA testing, HPV-associated proteins i.e. E6 and E7 mRNA expression, studied the potential role and application of cellular biomarkers, such as P16 (cyclin-dependant kinase inhibitor), Ki-67 (a core nuclear antigen for determining cell proliferation state), topoisomerase II-alpha (TOP2A) and minichromosomal maintenance-2 (MCM2), as diagnostic tools for early detection and prevention of the disease (Del Moral-Hernández et al., 2021; Gothwal et al., 2021; Guo et al., 2011; Li et al., 2019; Orang'o et al., 2020; Peres et al., 2016; Popiel et al.,

2021; Ren et al., 2019; Shi et al., 2019; Song et al., 2020, 2021; H.-W. Wang et al., 2011; Zuberi et al., 2021).

2.5 Role of P16 Immunohistochemical Over-Expression in Early Detection of Cervical Cancer

P16 is a cyclin-dependant kinase inhibitor and plays a crucial role as cell cycle regulator by decelerating cell progression from G1 to S phase. Low expression levels of P16 is observed in normal healthy cervical cells while it is overexpressed in both HSIL and cancer cells. Usually, P16 inhibits Cyclin Dependant Kinase 4/6 (CDK 4/6), which is responsible for phosphorylation of retinoblastoma protein (pRB). Its phosphorylation results in dissociation of pRB from E2F (a transcription factor), once dissociation occurs E2F enters into the nucleus and promotes targeted gene transcription necessary for cell transition from G1 to S phase. Therefore, P16 act as tumor suppressor by inhibiting CDK4/6. In HPV infected cells, E7 oncoproteins encoded by the virus incorporates into the host genome and binds with retinoblastoma protein. This binding leads to inactivation of pRB, thus E2F is liberated from P16 sequestration, entering into the nucleus, and as a result cell progress from G1 to S phase occurs. Hence, this functional loss of pRB causes reflex P16 overexpression through a negative-feedback mechanism (Buj & Aird, 2019; Rayess et al., 2011; Serra & Chetty, 2018). Consequently, P16 overexpression is directly linked to E7 oncoprotein activation of high-risk HPV serotypes, and can be used as a suitable marker for persistent high-risk HPV infection in vivo.

2.6 TOP2A Immunohistochemical Over-Expression and Cervical Cancer

TOP2A or Topoisomerases type IIA is one of the member of topoisomerase II enzyme family. This enzyme is responsible for DNA strands uncoupling during its replication and expressed only in cycling cells. This enzyme is important for double-strands breaks of DNA and stimulates gene transcription during M-phase (mitosis) of cell cycle (Goyal et al., 2020). The overexpression of this marker has been reported in a number of cancer including colon, cervix, and breast, where it induces increase cellular proliferation, tumor invasion and inhibit programmed cell death (apoptosis). TOP2A overexpression in cervical cancer was observed during transformation of low-grade intraepithelial lesion to high-grade intraepithelial lesion and cervical cancer (Sung et al.,

2021). The exact mechanisms and role of this enzyme in the initiation and advancement of CC is still not known. However, in a recent study, TOP2A overexpression was reported in CC tissue and the study concluded that its overexpression results in cell migration, tumor invasion and epithelial mesenchymal transition through PI3K/AKT signalling pathway activation (B. Wang et al., 2020).

2.7 Immunostaining of Several Biomarkers and Cervical Cancer

One study H.-W. Wang et al. (2011) evaluated the expression of ProExC (MCM2/TOP2A antibody Cocktail), P16, and Ki-67 biomarkers in 28 Formalin-Fixed, Paraffin-Embedded (FFPE) cervical cancer tissues using Immunohistochemistry (IHC). It was concluded that ProExC is a more sensitive proliferative biomarker for diagnosing cervical cancer compared to Ki-67, whereas P16 is highly specific to cervical carcinoma. Another study by Yang et al. (2013) concluded that immunohistochemistry of MCM2/TOP2A antibody Cocktail, P16 and Ki-67 has a higher sensitivity and specificity for interpretating cervical histopathology, and IHC assisted diagnosis has a higher consistency rate as compared to the original H&E diagnosis. A similar study by Peres et al. (2016) investigated the effectiveness of TOP2A (a component of ProExC) and Ki-67 using Immunocytochemistry (ICC) on 110 cervical smears. While it was concluded that both markers have the ability to effectively identify abnormalities as the lesions progress in severity, TOP2A was highly efficient in detecting high-grade lesions, whereas Ki-67 showed greater effectiveness in detecting atypical squamous cells and low-grade lesions by indicating the cell proliferation.

Another study by Guo et al. (2011) explored the efficacy of P16 and ProExC in detecting CIN 2+ lesions using IHC on 136 FFPE cervical tissue samples, and suggested greater sensitivity of P16 immunostaining in detecting CIN2+ lesions as compared to ProExC, which demonstrated a higher specificity in CIN3+. A more recent study by Zuberi et al. (2021) analyzed total 145 cervical cancer tissues using IHC to evaluate the diagnostic and prognostic capabilities of both P16 and TOP2A. It was noted that although the increased overexpression of TOP2A is associated with the disease progression, yet it is a less reliable indicator of cervical cancer prognosis as opposed to P16, which is not only linked with the degree of histological dysplasia and malignancy but can also be used as a valuable prognostic and predictive marker. A recent study by Del Moral-Hernández

et al. (2021) has analyzed the expressions of various biomarkers, including TOP2A/MCM2, P16, cyclin E1, telomerase, and DNA oxidative damage markers, using ICC in more than 1450 non-invasive LBC samples diagnosed as CIN and/or biopsy-confirmed cervical cancer cases. This study too has suggested high sensitivity and precision of the mentioned biomarkers in identifying cervical lesions with a greater risk of progressing to cervical cancer.

A study by Song et al. (2020) has explored the utilization of P16 immunocytochemistry as a screening tool for cervical cancer. The outcome suggested that P16 staining has proven to be highly accurate when used as both primary and secondary screening method and showed a noticeable advantage in triaging primary HPV or LBC screening. In the later study conducted in 2021 he further explored P16 immunocytochemistry in more than 2700 women with extended HPV genotyping and concluded that the combination of both is more effective compared to relying merely on HPV genotyping in triaging the probable cases.

Another recent study by Shi et al. (2019) has evaluated the expression level of P16 and Ki-67 proteins in premalignant and cancer cases using IHC. It was found that the expression level of both proteins was substantially higher in high-grade CIN and cancer cases as compared to low-grade CIN and control groups. The study concluded that the overexpression of P16 and Ki-67 proteins is linked with the advanced stages of cervical lesions. A similar study by Li et al. (2019) assessed the utilization of P16/Ki-67 co-testing alone and in conjunction with HPV DNA loads in 271 cases using IHC. It was noted that combining P16/Ki-67 co-testing with HPV DNA loads is effective in predicting the outcome of CIN-2 lesion in patients infected with HPV-16 and/or 58 genotypes. Another similar study by Ren et al. (2019) determined both diagnostic and predictive value of P16/Ki-67 immunocytochemistry, HPV E6/E7 mRNA testing, and HPV DNA assay in 103 women with atypical squamous cells of undetermined significance (ASC-US). It was deduced that high expression of HPV E6/E7 mRNA can be useful for the diagnosis, while P16/Ki-67 immunocytochemistry also exhibited the ability for triaging ASC-US cases.

Researchers have also explored the effectiveness of Visual Inspection with Acetic acid (VIA), HPV DNA testing, and P16/Ki-67 dual cytology using ICC in 700 women

for CC screening (Orang'o et al., 2020). It was found that high-risk HPV infections have 2-fold higher prevalence among women with HIV co-infection. Moreover, VIA demonstrated low sensitivity in comparison to P16/Ki-67 dual cytology which exhibit both higher sensitivity as well as specificity in detecting high-grade cervical lesions. A similar study by Gothwal et al. (2021) examined the use of P16/Ki-67 biomarkers to identify dysplastic cervical lesion and triage abnormal cytological smears using ICC. It was noticed that P16/Ki-67 dual cytology staining increased with advancing cytological abnormality and showed 100% positivity rate in HSIL and cancer cases, implying that P16/Ki-67 dual cytology staining can enhance the positive predictive value for the diagnosis and detection of transforming HPV infections, contributory to the higher sensitivity as well as specificity values in detecting CIN2+ lesions.

Another study concluded that in HPV positive women P16/ki67 dual staining is a reliable maker for the diagnosis of abnormal cytological lesions and to prevent colposcopy referrals unnecessarily (Aromseree et al., 2022).

A latest study by Ssedyabane et al. (2024) measured the serum concentration of P16ink4a in precancerous, cancerous and control groups by quantitative ELISA and higher concentration was observed in CC as compared to CIN and control group which suggests that there is an association between serum P16ink4a levels and cervical lesions.

Another study assessed P16 immunoreactivity via IHC in benign, premalignant and invasive SSC and adenocarcinoma of cervix and it was shown that P16 expression was absent in benign cervical tissue whereas P16 overexpression was directly related to the degree of cervical dysplasia (Kishore & Patil, 2017).

A similar study from South-India also assessed the immunohistochemical expression of P16 in 150 CC tissue and found that more than 90% of the cases were hr-HPV positive. Moderate P16 expression level was noted in one-third of these case while rest of the two-third hr-HPV positive cases showed P16 overexpression. The study concludes that P16 overexpression increases with the high-risk HPV viral load in the host genome (Sujatha et al., 2022).

Another recent study evaluated various biomarker i.e. P16, MIB-1 and CK-17 on cervical benign, CIN and cancer tissues via immunohistochemistry and showed overall good sensitivity, specificity, PPV and NPV of these markers and suggest utilization of these marker in routine histopathology reporting for accurate diagnosis (Sahu et al., 2022).

Another study evaluated different immunohistochemical biomarkers panel including P16, CK-17, P63 and HPV testing to distinguish CIN from its mimics. The findings showed higher sensitivity and specificity for P16 as compared to HPV, however P63 and CK-17 did not demonstrate any significance in identifying CIN mimics (Selvi et al., 2014). However, a recent study by Iranpour et al. (2021) had contradictory results where these three biomarkers i.e. P16, P63 and CK-17 showed significant immunostaining in CIN lesions and can be a reliable biomarkers panel to distinguish CIN from its mimics.

In another prospective study by White et al. (2020) total 275 HPV positive women were assessed for P16/Ki-67 dual immunocytochemistry along with urinary nicotine metabolite evaluation and concludes that the probability of high-grade lesions are 2-fold higher among women who are subjected to tobacco smoke and are at increased risk of dual P16/Ki-67 staining positivity.

2.8 Molecular Biomarkers and Cervical Cancer

In the past few years, many researchers and scientists have also investigated the potential role of various molecular biomarkers and multiple genetic studies have been conducted for cervical cancer screening. A recent study by Giorgi Rossi et al. (2021) assessed the accuracy of P16/Ki-67 immunostaining along with HPV mRNA expression in triaging HPV DNA-positive cases. These patients underwent additional tests and were referred for colposcopy or repeat testing after a year randomly. The results suggest that utilization of both P16/Ki-67 immunostaining and HPV mRNA expression will aid in triaging HPV DNA positive women and can improve high grade lesion identification in these patients. Therefore, improving the accuracy of cervical cancer screening and management of HPV DNA positive cases.

Another recent study by Jin et al. (2023) retrospectively analyzed 1387 women with cytological diagnosis of ASC-US and HPV E6/E7mRNA positivity. The analysis included LBC examination and detection of specific HPV genotypes in both premenopausal and postmenopausal groups. The results indicated different HPV genotype identification in ASC-US. Therefore, this study gives an insight on HPV prevalence among women with cytological diagnosis of ASC-US and emphasises the crucial role of HPV testing for the management of patients with ASC-US.

Another study by Tüney et al. (2017) evaluated HPV genotypes and HPV E6/E7 mRNA expression in LBC samples among Turkish women with abnormal cytological results. HPV DNA and E6/E7 mRNA molecular testing were performed using commercial assays. Total 81 women with abnormal cytology results were included in the study and the results showed that in a subset of samples, HPV DNA was identified specifically HPV 16 genotype, along with specific HPV E6/E7 mRNA levels for various high-risk genotypes i.e. 16,18,31,33, and 45 were detected. These findings indicate that the E6/E7 mRNA molecular testing is a reliable indicator of cytological atypia and correlates better with advancing lesions than HPV DNA assays.

A similar study evaluate HPV E6/E7 mRNA performance and compare it with HPV DNA testing and LBC. The study showed that mRNA assay has equivalent sensitivity as HPV DNA testing but its specificity for detecting high grade lesion CIN2 or CIN2+ is higher in contrast to HPV DNA testing whereas lower than LBC. Therefore HPV mRNA assay could be a potential biomarker for early detection of cervical lesions and lower the rate of false positive results (S.-K. Zhang et al., 2020).

A similar study in China evaluated the performance of HPV E6/E7 mRNA assay as a primary screening modality and triage for cervical cancer and it was observed that the mRNA assay has higher sensitivity than LBC whereas low specificity as compared to LBC and the author concluded that the application of mRNA genotyping assay in conjunction with LBC testing could be an effective and feasible approach to triage women for early cervical cancer detection (J. Zhang et al., 2022).

A recent study by Huo et al. (2020) explored the expression of P16INK4a, Notch1, and hTERC genes and the protein expression levels using RT-PCR and western blot. The study concluded that P16INK4a gene can be used as an early screening biomarker of cervical cancer, and the hTERC gene can be used to verify the clinical diagnosis of cervical cancer. In another study A. Wang et al. (2021) assessed the expression of miR-29a and methylated status of P16 promoter. The results exhibited that miR-29a expression was downregulated in cervical cancer tissues and cells, and negatively correlated with P16 promoter hypermethylation. A study by Q. Zhao et al. (2020) used three raw microarray datasets, 188 differentially expressed genes (DEGs) were identified. It was found that TOP2A may a probable tumor oncogene and a biomarker for the prognosis of cervical cancer. A similar study by Yu et al. (2020) identify master regulators (MRs) using transcriptome data in cervical cancer. It was found that TOP2A and CENPF are a synergistic pair of MRs that are activated and overexpressed in Cancer cases. Their high expression is correlated with some prognostic and the molecular features and was noticeably high in CC and can be good biomarkers and anticancer drug targets for CC.

In a recent study J. Zhang et al. (2024) reported that in high-risk HPV-16 positive individuals, E6 oncoprotein regulates the level of miRNA-320a which promotes cell proliferation, migration, invasion and blocks apoptosis in cervical cancer. It was also found that TOP2A is one of the downstream targeting protein of miRNA-320a leading to the development of CC. Another similar study evaluated the miRNA-9-5p expression level in Squamous Cell Carcinoma (SCC) of cervix by quantitative q-PCR and concluded that its overexpression is correlated with an increase migration and invasion ability, and enhance Epithelial-Mesenchymal Transition (EMT) process of cervical cancer cells (Kuang et al., 2023).

Epithelial-Mesenchymal Transition (EMT) phenomena is an important event observed in metastatic phase of cancer particularly in CC where epithelial phenotype of the cells are replaced by mesenchymal features. In a recent study EMT markers TWIST, SNAIL and SLUG were analyzed via immunohistochemistry in pre-cancerous and CC lesions. It was found that overexpression of these proteins were more pronounced in high-grade and cancerous lesion as compared to low-grade and control groups. The study proposed utilization of these EMT markers in combination with other well-known

cervical cancer markers in order to improve the diagnostic outcomes (Popiel-Kopaczyk et al., 2023).

In summary, numerous research studies have investigated the efficacy of various biomarker for early detection, progression and prognosis of cervical cancer. While these rapid technological advancements have ensured promising results, yet further studies are needed to develop cost-effective tools for easier implementation in the low and middle income countries with significantly higher proportion of the cases as compared to the developed nations.

2.9 Future Directions for Cervical Cancer Screening

2.9.1 Emerging Biomarkers for Cervical Cancer Screening

The landscape of cervical cancer screening is expected for substantial revolution, driven by continuing researches into novel biomarkers and advanced technological developments. These novelties can augment both the sensitivity and specificity of screening strategies, thus improving early detection of the cervical lesions and patient's prognosis. At present, many scholars are actively exploring a variety of novel biomarkers that could transform cervical cancer screening. Methylation markers are one of the promising markers, which functions by adding a methyl group to the DNA. These methylation changes in the DNA can specify the existence of cervical pre-cancerous and cancerous lesions and aid as an accurate diagnostic tool. Additionally, researchers are investigating different protein expression in cervical cells and identify specific protein related to cervical cancer.

2.9.2 Emerging Technologies in Cervical Cancer Screening

Besides application of various biomarkers, technological advancements are also significantly contributing in cervical cancer screening protocols upgradation. Advanced imaging techniques i.e. enhanced colposcopy assists in remarkable visualization of cervical tissue, thus helps in early detection of intraepithelial lesions. Another emerging technique NGS-next generation sequencing is being explored for cervical cancer screening. Detailed analysis of genetic material and information regarding genetic

mutations and alterations that cause cancer development can be investigated by NGS technique. NGS not only offers enhanced screening accuracy but also identifies high-risk groups, hence allowing for more tailored screening strategies. Moreover, Artificial Intelligence (AI) is now being explored in cervical cancer screening protocols. These algorithms can assist cytologist in examining extensive datasets and detect cervical cell abnormalities for accurate diagnosis of cervical lesions.

The upcoming decade of cervical cancer screening is anticipated to be characterized by an integrated approach that incorporates several biomarkers and advanced technologies. This comprehensive approach offers a more robust and accurate screening process, which leads to an earlier identification of cervical intraepithelial lesions, early management, and better patient's prognosis.

2.10 OPERATIONAL DEFINATION[9]

For reporting cervical cytology samples, The Bethesda System (TBS) 2014 was applied.

2.10.1 Cervical Cell Adequacy

8,000 to 12,000 well preserved, well visualised squamous cells on a conventional smear or 8 to 10 cells per high power field (40X) will be considered adequate with no obscuring of cells with blood or inflammatory cells.

2.10.2 Atypical Squamous Cells (ASC) of Undetermined Significance (ASC-US)

Cytomorphologic features of LSIL are not always conclusive and there are some grey-area findings that are questionable but not conclusive for LSIL and fall into the category of ASC-US.

2.10.3 Atypical Glandular Cells (AGCs)

Glandular cells with morphology, either quantitatively or qualitatively falls short of interpreting as endocervical adenocarcinoma in situ or invasive adenocarcinoma (Bethesda 2015). Criteria for AGCs includes Cells occurring in sheets and strips, with nuclear crowding, overlap, and/or pseudo-stratification, Cell groups resembling rosettes/ or forming glands or feathering, Enlarged and elongated nuclei with hyperchromasia, Coarse chromatin with heterogeneity, Occasional mitoses and/ or apoptotic bodies, Increased nuclear-cytoplasmic ratio, Ill-defined cell borders.

[9] Alrajjal A, Pansare V, Choudhury MSR, Khan MYA, Shidham VB. Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of Uterine Cervix and Bethesda System. Cytojournal. 2021 Jul 17; 18:16. doi: 10.25259/Cytojournal_24_2021. PMID: 34345247; PMCID: PMC8326095.

2.10.4 Atypical Squamous Cells (ASC) cannot exclude HSIL (ASC-H)

LSIL with immature metaplastic cytoplasm can be difficult to differentiate from ASC-H or HSIL. ASC-H is entitled for cases with ambiguous cellular changes for high-grade dysplasia due to quantitative or qualitative limitations.

2.10.5 LSIL (encompassing HPV/Mild Dysplasia/CIN 1)

LSIL refers to morphological changes at the lower end of the SIL spectrum includes CIN-1. LSIL is a lesion of intermediate or superficial cells that shows nuclear enlargement accompanied by moderate variation in nuclear size and slight irregularities in nuclear shape and contour. The important dysplastic features includes nuclear enlargement (3x intermediate cells), multinuclearity, hyperchromasia, and perinuclear halos.

2.10.6 HSIL (encompassing Moderate and Severe Dysplasia/ CIS/CIN 2 and CIN 3)

HSIL refers to morphological alterations associated with the high end of the SIL spectrum and including both CIN-2 and CIN-3. Nuclear atypia is characteristic feature of HSIL. HSIL cells are smaller than LSIL in both cytoplasmic volume and nuclear size and have a higher N/C ratio. However, the nuclei display important dysplastic features such as hyperchromasia, coarse but delicate chromatin, and nuclear envelope irregularities with no nucleolar prominence.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study Design

This research was a cross-sectional study based on the analysis of cervical cytology samples. This study commenced after getting approval from the Institutional Review Board (IRB) of Bahria University Health Sciences Campus (BUHSC) Karachi and Dow University of Health Sciences (DUHS).

3.2 Subjects

This study was conducted on high-risk groups coming to Gynaecology OPD for cervical smear.

3.3 Place of Sample Collection/Study Setting

PNS Shifa Hospital, Karachi and Dr. Ruth K. M. Pfau, Civil Hospital, Karachi

3.4 Inclusion Criteria

- Married Women of aged between 25–65 years
- All cervical samples taken from high risk group with normal, pre-cancerous lesions and early-stage cancer on cytology reporting

High risk group includes patients with HPV infection, low socio-economic status, smoking, weak immune system, prolonged use of oral contraceptives, early marriage before age 18 years, early sexual activity, multiple sexual partners, multiple sexual

partners of spouse, and multiple childbirths. History of irregular bleeding per vaginum, foul smelling vaginal discharge, post coital bleeding and/or post-menopausal bleeding as well as patients with cervical lesions and known cases of endocervical cancer.

3.5 Exclusion Criteria

- Pregnant women
- Women with other STD infection currently
- Women who had prior history of radiotherapy or cervical resection
- Women with history of hysterectomy
- Inadequate sample

3.6 Duration of Study

From 1st November 2023 to 30th June 2024

a) Individual study duration: 3-5 days

b) Total duration of study: 8 months

3.7 Sample Size Estimation

The total sample size of this research study was 60. The sample was divided into pre-cancerous (LSIL & HSIL), early stage cervical cancer and normal subjects.

Sample size was calculated from OpenEpi (Version 3) open source calculator by assuming 95% confidence interval with 5% margin of error. The calculated sample size was obtain to be 60 based on the prevalence of Cervical Cancer in Pakistan taken from Shaukat Khanum Collective Cancer Registry Report – Dec. 1994 to Dec. 2022.

Sample Size for Frequency in a Population

Population size (for finite population correction factor or fpc) (N): 1000000

Hypothesized % frequency of outcome factor in the population (p)[10]: 4%+/-5

Confidence limits as % of 100 (absolute +/- %) (d): 5%

Design effect (for cluster surveys-DEFF): 1

Sample size n = [DEFF*Np(1-p)]/ [(d2/Z21-α/2*(N-1)+p*(1-p)]

Sample size calculated = 60

3.8 Sampling Technique

Non-probability Consecutive sampling technique.

3.9 Human Subjects and Consent

Informed consent was obtained from each participant prior to sample collection by using the designed Consent form.

3.10 Materials

3.10.1 Questionnaire

Each participant was asked regarding the demographics, general biodata, risk stratification and clinical features using the designed Performa. However, the cytological examination and immunohistochemical findings analysis were documented on the designed cytological form.

3.10.2 Culture Media

N/A

[10] Shaukat Khanum Collective Cancer Registry Report – Dec. 1994 to Dec. 2022

3.10.3 Drugs

N/A

3.10.4 Equipment's, Materials and Reagents used

- PathTezt EasyVial liquid based cytology kits
- Cervical Cytology Brush (CB1-10100)
- Papanicolaou (PAP) stain for all cases
- Human plasma and Thrombin for cell block preparation
- 10% Buffered Formalin for cell pellet fixation
- Poly-L-lycine coated glass slides for immunocytochemical markers
- Antigen Retrieval Solution
- Blocking Agent
- Primary Antibodies P16 and TOP2A
- CDKN2A/P16-INK4a: (Monoclonal), Mouse (IgG) Monoclonal antibody (MA5-17054), Thermofisher Scientific
- Topoisomerase II alpha: (Monoclonal), Rabbit (IgG) Monoclonal antibody (MA5-36063), Thermofisher Scientific
- Secondary antibody Horseradish Peroxidase (HRP)
- Enzyme-labelled polymer system
- Chromogen substrate
- Hematoxylin for counterstain
- Phosphate buffer solution

3.11 Parameters of Study

- This study was conducted to assess the expression levels of P16 and TOP2A by Immunohistochemical staining on selected Formalin Fixed Paraffin Embedded (FFPE) cervical cytology cell blocks of normal, cervical pre-cancerous and cancerous specimens.
- Clinical history in order to collect data regarding patient's age, demographic data, risk stratification and clinical diagnosis was obtained with the help of designed Performa.
- Papanicolaou (PAP) stained slides of the selected cases was reviewed by two senior histopathologists to gather information regarding cervical cell adequacy, morphology and diagnosis.
- P16 was expressed as brownish-yellow only nuclear staining or both the nucleus and cytoplasm immunostaining.
- TOP2A was expressed as brownish-yellow only nuclear immunostaining.

3.12 Study Protocol

- After getting the approval by IRB of both Institutes and FRC, written informed consent form was signed from each participant selected on the basis of inclusion criteria
- Clinical history and appropriate patient's data was collected using the designed Performa to select the high-risk group
- Previous cytology reports were reviewed (if available)
- Vaginal and speculum examination was conducted before collecting the cells from transformational zone of cervix
- Liquid-based cytology kits were used for collection, transportation and cell block preparation of cervical cells
- Collection of the cells was done with the help of cervical cytology brush (CB1-10100) following standard guidelines

- The collected cells were spread onto a glass slide and fixed using a fixative solution
- Two senior histopathologists examined PAP stained slides for cell adequacy, morphology and diagnosis
- Residual cervical cytology sample was used for making cell blocks
- Immunohistochemical (IHC) staining was executed on Formalin Fixed, Paraffin-Embedded cell block sections according to the manufacturers' protocols
- All slides were examined under a light microscope scanner (10x10), and high power lens (40x10) and were reviewed by the supervisor.
- Statistical analysis of the results was done using IBM SPSS version 27

3.12.1 Cell Block Preparation

- Cell blocks were made from the residual liquid-based cytology samples via plasma thrombin method as shown in Figure 3.1
- After fixation of samples for a few hours, the container was centrifuged to separate the cellular material from the liquid component
- Supernatant was carefully removed without disturbing the cell pellet
- Excess supernatant was dried using the filter paper
- 3 drops of plasma was added and gently stirred with the help of wooden stick to permit plasma permeate through the sediment (cell pellet)
- Then 3 drops of thrombin via syringe was added to cell pallet and gently stirred with the help of wooden stick to permit thrombin permeate through the sediment (cell pellet)
- After formation of clot, it was transferred to a container with 10% buffered formalin for further fixation

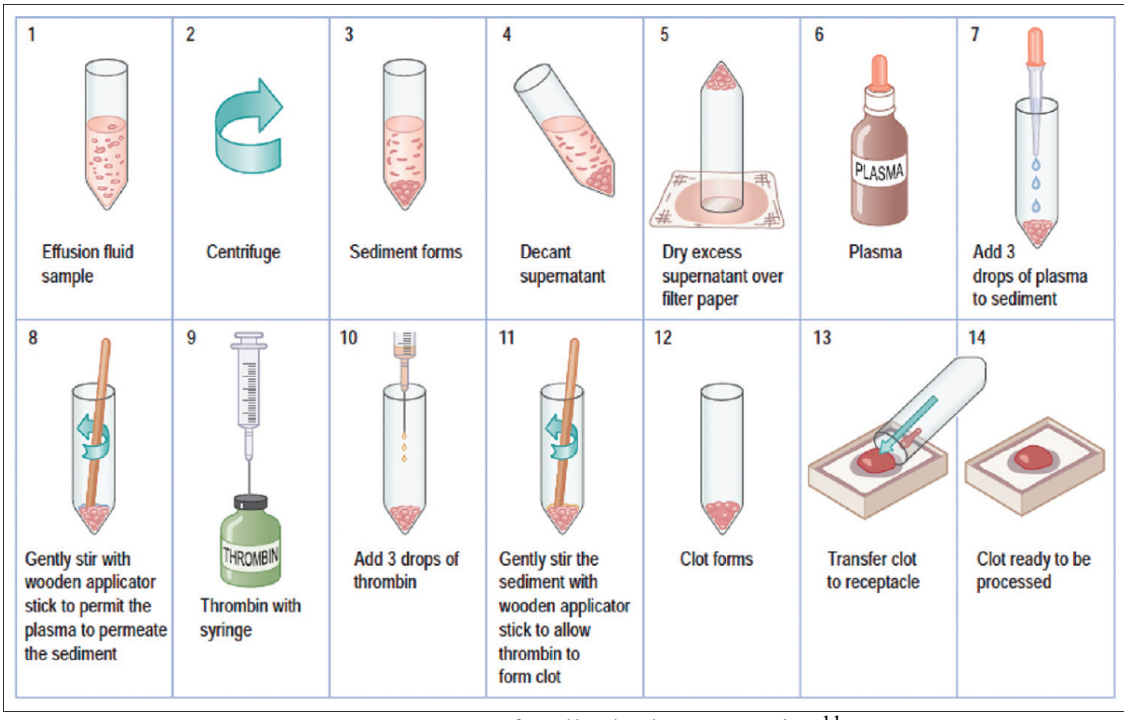


Figure 3.1 Steps of Cell Block Preparation[11]

- Processing of the formalin-fixed cell pellet was done using routine histological techniques i.e. dehydration, clearing, embedding in paraffin, sectioning, and mounting onto glass slides

- The sections mounted on glass slides were stained using H&E staining. Immunohistochemical stains were subsequently applied to identical sections, followed by coverslip mounting and sealing.

3.12.2 Methods of Staining

3.12.2.1 Papanicolaou's Staining

Cytology cells were proceeded through numerous solutions as shown in Figure 3.2 and 3.3.

a) Fix cervical cytology cells in 95% Ethanol --- 15 min

[11] Protocol for plasma-thrombin method (Reproduced from: Shidham and Atkinson, 'Cytopathologic Diagnosis of Serous Fluids' Chapter #14 (Appendix 1), Elsevier (W. B. Saunders Company) First edition, 2007 (ISBN-13: 9781416001454[12])

b) 70% Ethanol --- 1 min

c) 50% Ethanol --- 1 min

d) Distilled water --- 5 dips

e) Harris haematoxylin --- 3 and half minutes

f) Distilled water ---5 dips

g) 0.25% aqueous solution of Hydrochloric acid ---few dips

h) Water --- 1 min

i) Lithium carbonate --- 1 and half minute

j) Water --- few dips

k) 70% Ethanol --- 2 min

l) 90% Ethanol --- 2 min

m) Orange G --- few dips

n) 95% Ethanol --- 2 min

o) EA modified --- 2 min

p) Absolute Ethyl alcohol – 2 changes --- 2 min each

q) Xylene – 2 changes --- 5 min or until cleared

r) Mounting in DPX

- **Result:**
  - Nuclei: Dark blue
  - Cytoplasm: Blue green

(Reference: Pranab Dey (2018) Basic and Advanced Laboratory Techniques in Histopathology and Cytology)

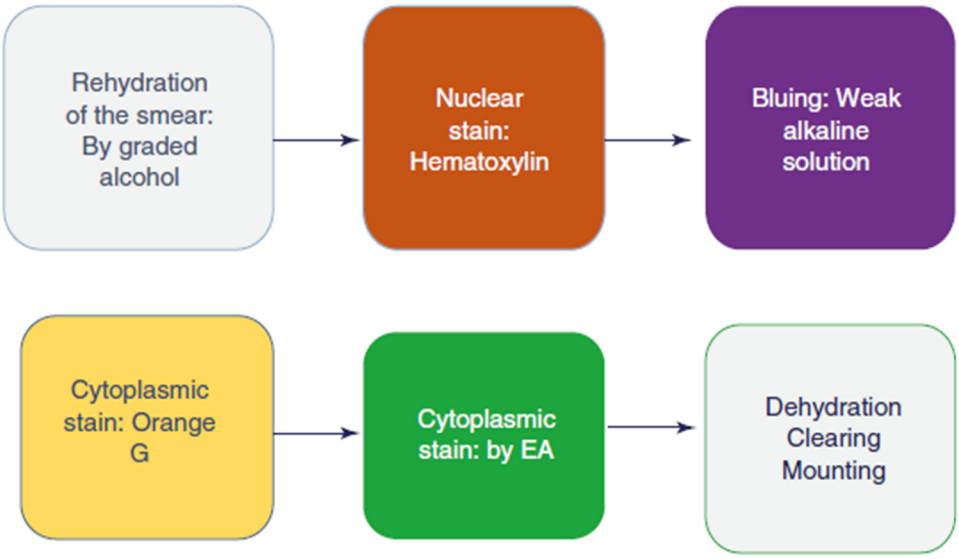


Figure 3.2 Principles of Papanicolaou's (PAP) Stain[12]

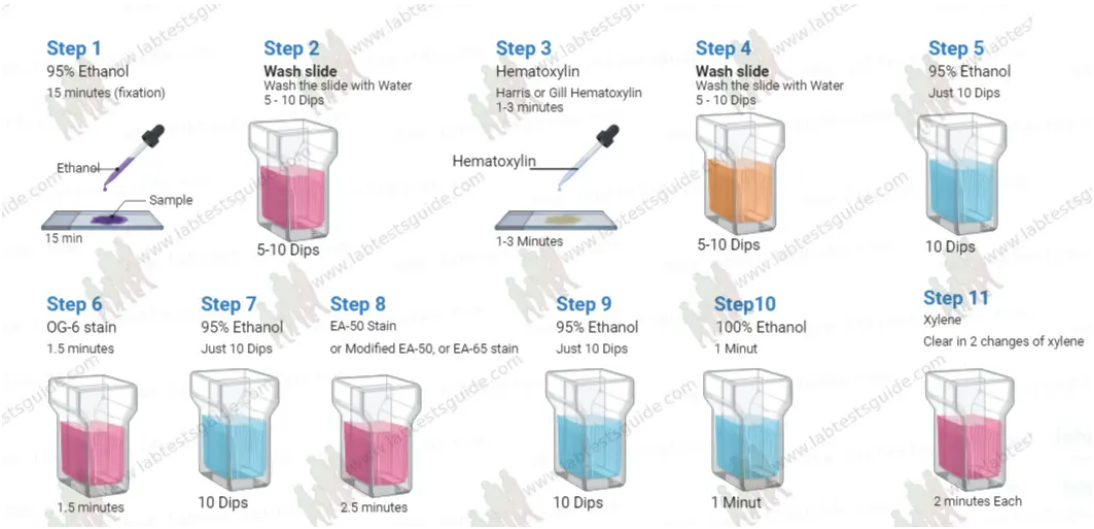


Figure 3.3 Steps of Papanicolaou's (PAP) Stain[13]

3.12.2.2 Haematoxylin and Eosin (H&E) Staining

Sections proceeded through numerous solutions as shown in Figure 3.4 and 3.5.

a) Xylene I --- 10 min

[12] Pranab Dey (2018) Basic and Advanced Laboratory Techniques in Histopathology and Cytology
[13] Lab Test Guide (28th May 2023) https://www.labtestsguide.com/pap-stain

b) Xylene II--- 10 min

c) Absolute Alcohol-- 10 min

d) 95% Alcohol --- 5 min

e) 80% Alcohol --- 5 min

f) 70% Alcohol --- 5 min

g) Tap water rinse -- 2 min

h) Haemtoxylin ---- 5-10 min

i) Acid Alcohol (1% HCl in 70% alcohol) for differentiation , 3-5 dips then washed with tap water

j) Ammonia water, 3-5 dips then rinsed with tap water for 10 min

k) Eosin --- 2 min

l) 70% Alcohol --- 5 quick dips

m) 80% Alcohol -- 5 quick dips

n) 95% Alcohol --- 5 quick dips

o) Absolute Alcohol – 2 changes – 5 min each

p) Xylene --- 5 min

q) Mounted in Dako Toluene free mounting media

- **Results**

  - Nuclei stained blue
  - Cytoplasm stained varying shades of pink

(Reference: Kiernan,J.A (2008) Histological and Histochemical Methods: Theory and Practice. 4th ed. Bloxham, UK)

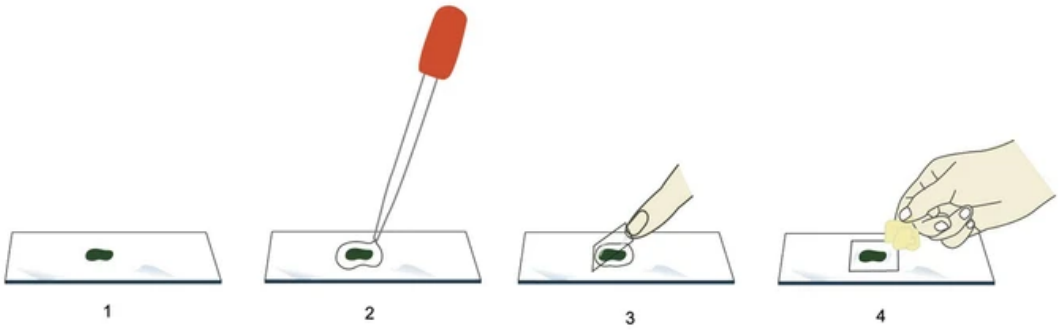


Figure 3.4 Steps of Slides Mounting

3.12.2.3 Immunohistochemical Staining

- **Primary Antibodies**

1. CDKN2A/P16-INK4a Mouse (IgG) Monoclonal antibody

    - Localization: Cytoplasm and Nucleus
    - Positive Control Tissue : Cervical Squamous Cell Carcinoma and melanoma
    - Dilution : 1: 200

2. Topoisomerase II alpha: (Monoclonal), Rabbit (IgG) Monoclonal antibody

    - Localization: Nucleus
    - Positive Control Tissue: Breast carcinoma, Small cell lung Carcinoma and testicular seminomas
    - Dilution: 1: 100

- **Procedure**

➢ Immunohistochemical staining was performed on selected pre-cancerous and cancerous cervical specimens as shown in Figure 3.6 and 3.7

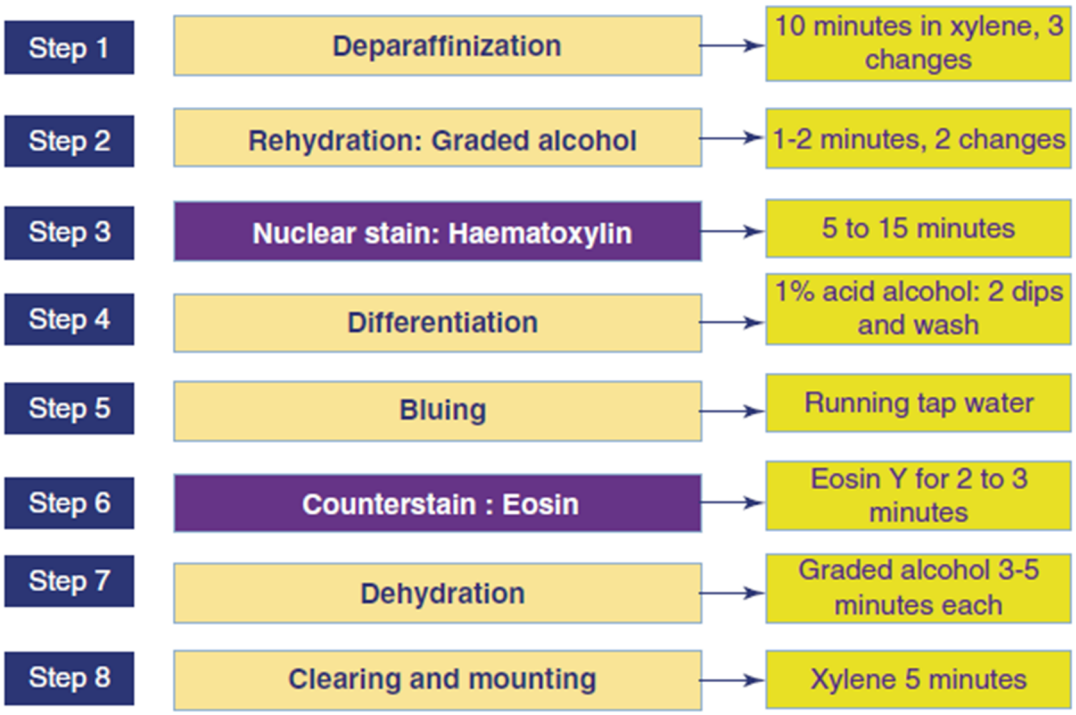


Figure 3.5 Steps of Haematoxylin and Eosin (H&E) Stain[14]

- Thin Sections of about 2-4 micrometre thickness were taken from Formalin Fixed Paraffin Embedded cell blocks and were collected on poly L-lysine coated slides.
- Slides were fixed in oven at 80°C for 20-25 min

The procedure involves several steps:

Deparaffinization, Hydration and Antigen Retrieval

- Deparaffinization, hydration, and antigen retrieval were done automatically through Dako PT Link pretreatment system in 40-45 minutes
- Initial temperature was 65°C when the slides were kept in, then it was raised to, and kept at 97°C for 40 min, then the temperature was lowered to 65°C, and then slides were taken out
- Slides were then washed with washing buffer 2 times for 5 minutes each

[14] Pranab Dey (2018) Basic and Advanced Laboratory Techniques in Histopathology and Cytology

Blocking

- Endogenous peroxidase was blocked by 3% hydrogen peroxide solution
- On cell block sections 1-2 drops of blocking solution were added to cover the section
- Slides were incubated for 20 minutes in humidity chamber at room temperature
- Slides were washed with washing buffer 2 times for 5 minutes each

- **Primary Antibodies**

- Both P16 and TOP2A antibodies were diluted in the ratio of 1:200 and 1:100 respectively according to the manufacturer's protocol
- Dilution was done by antibody diluent
- Primary antibody was applied to cover the section along with the positive and negative controls
- Sections were incubated overnight in humidity chamber at 4°C
- Slides were then rinsed gently with washing buffer twice for 5 minutes each

- **Secondary Antibody**

- Enough Horseradish Peroxidase (HRP) was applied to cover the section
- Slides were incubated for 1 hour in a humidity chamber at room temperature
- Slides were then rinsed gently with washing buffer twice for 5 minutes each

- **DAB Substrate Chromogen**

- Diaminobenzidine (DAB) substrate chromogen was prepared with 1 ml of DAB substrate and 1 drop of DAB chromogen
- Each slide was allowed to drain and was wiped carefully

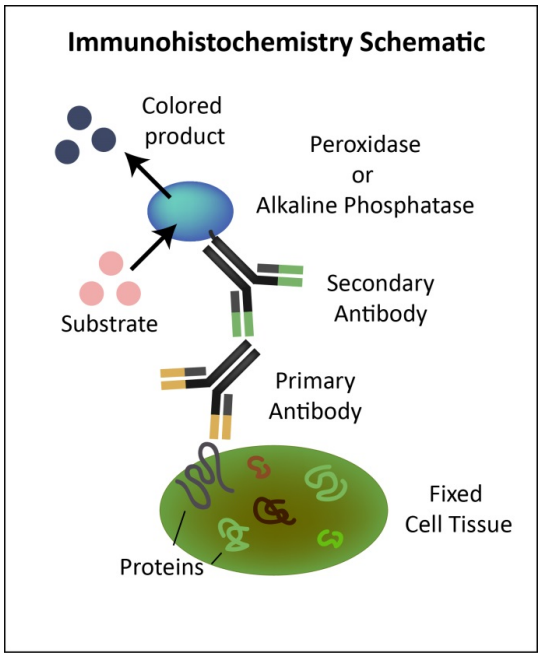


Figure 3.6 Immunohistochemistry Principle[15]

- Enough DAB substrate chromogen solution was applied to coat the section
- Slides were incubated for 1- 2 minutes at room temperature
- Slides were then washed with distilled water

- **Hematoxylin Counterstain**

- Slides were counterstained with Hematoxylin stain, 1-3 dips
- Slides were washed gently with washing buffer
- Sections were dehydrated in ascending series of Alcohol (60%, 80%, 100%) and cleared in Xylene
- Slides were mounted in Dako toluene free mounting media and a coverslip was sealed
- Slides were then observed under light microscope

[15] Immunohistochemistry (IHC) Protocol https://www.epigentek.com/catalog/ immunohistochemistry-ihc-protocol-n-19.html

3.12.3 Interpretation

- For P16 brownish-yellow only nuclear or both the nucleus and cytoplasm nuclear immunostaining was observed
- For each sample, 5 microscopic fields at 40x magnification will be selected, and 100 tumor cells in each field will be counted to access the staining intensity and percentage of positive cells
- The percentage of P16 positive tumor cells (A) was assessed in five gradation:

  0= no cells stained positive

  1= <10% of the cells stained positive

  2= 10 to 50% stained positive

  3=51 to 80% stained positive and

  4= >80% of the cells stained positive

and was multiplied by the staining intensity (B) quantified in four gradations:

no visible staining = 0

weak staining = 1

moderate staining = 2

and intense staining = 3

Final Immunoreactive IRS-score obtained by (AxB)

0-1 = negative

2-3 = mild expression

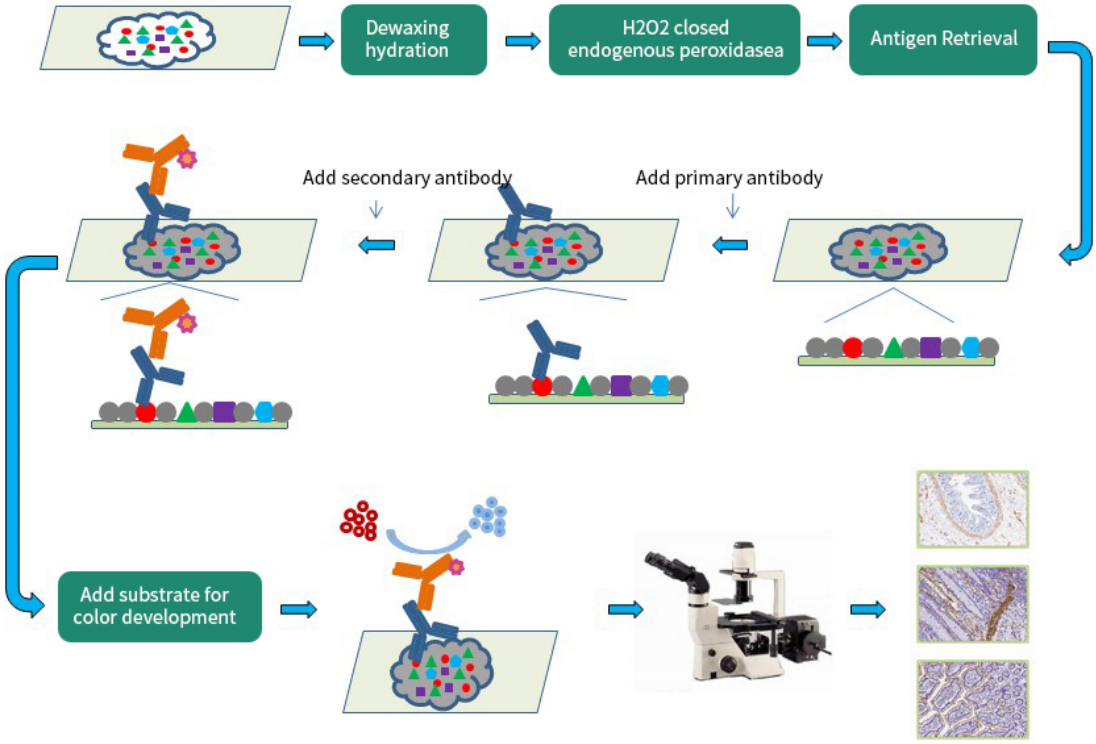


Figure 3.7 Immunohistochemistry Procedure[16]

4-8 = moderate expression

9-12 = strongly positive expression

Reference: N. Fedchenko and J. Reifenrath, 'Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue – a review', Diagn. Pathol., vol. 9, no. 1, p. 221, Dec. 2014, doi: 10.1186/s13000-014-0221-9.

- For TOP2A brownish-yellow only nuclear immunostaining was observed
- For each sample, 5 microscopic fields at 40x magnification will be selected, and 100 tumor cells in each field will be counted to access the staining intensity and percentage of positive cells.
- The percentage of TOP2A positive tumor cells (A) was assessed in five gradation:

  0= no cells stained positive

  1= <10% of the cells stained positive

[16] Immunohistochemistry (IHC) protocols https://www.sinobiological.com/category/ihc-protocol

2= 10 to 50% stained positive

3=51 to 80% stained positive and

4= >80% of the cells stained positive

and was multiplied by the staining intensity(B) quantified in four gradations:

no visible staining = 0

weak staining = 1

moderate staining = 2

and intense staining = 3

Final Immunoreactive IRS-score obtained by (AxB) with range (0-12)

0-1 = negative

2-3 = mild expression

4-8 = moderate expression

9-12 = strongly positive expression

Reference: N. Fedchenko and J. Reifenrath, 'Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue – a review', Diagn. Pathol., vol. 9, no. 1, p. 221, Dec. 2014, doi: 10.1186/s13000-014-0221-9.

3.14 Flow Chart / Algorithm of Study

The following figure shows the work flow of this research study:

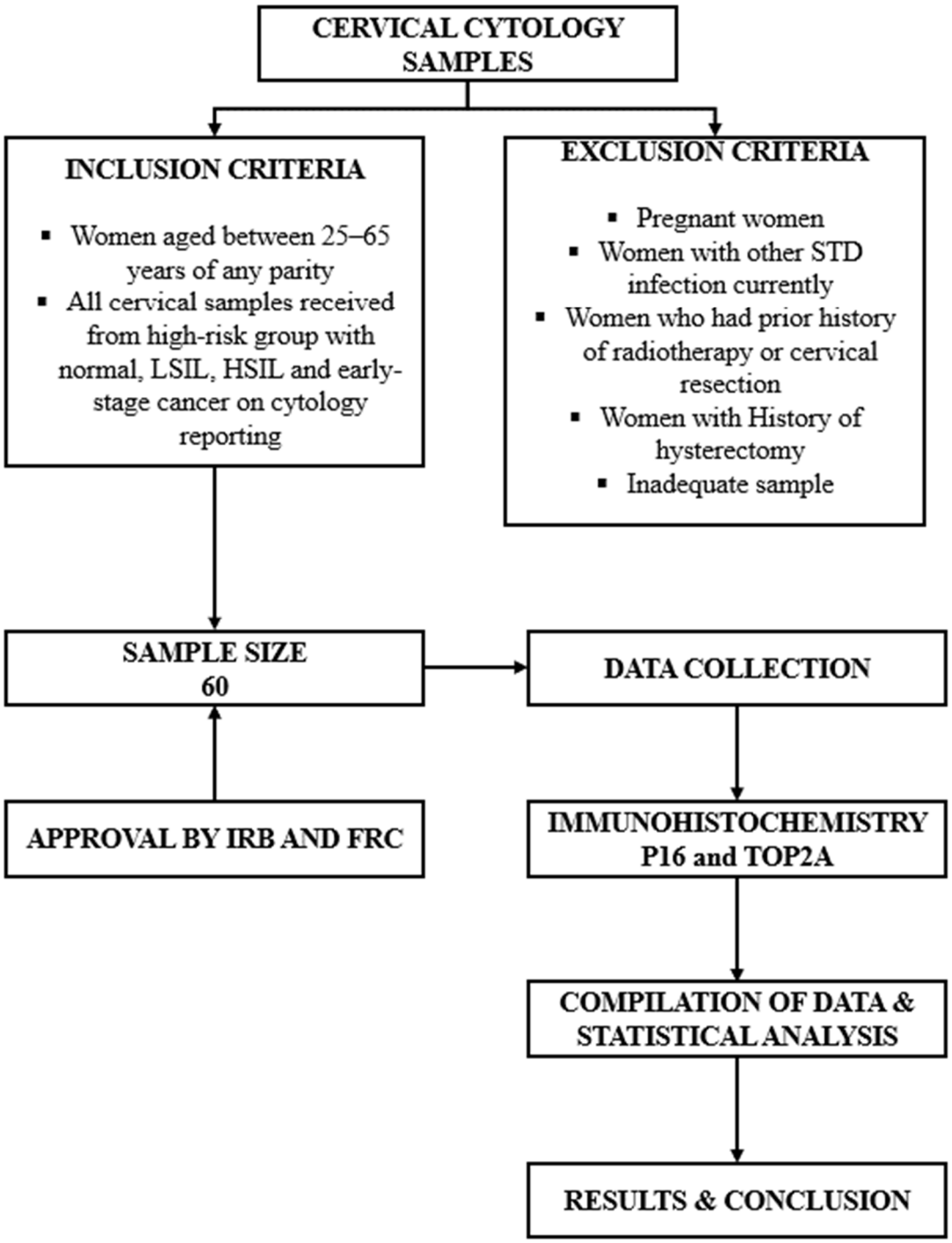


Figure 3.8 Work flow of study

3.15 Statistical Analysis

- All Statistical analysis was conducted using IBM SPSS Statistics version 27
- Mean and standard deviation were calculated for quantitative variables
- Frequency and percentages were calculated for qualitative variables
- Pie charts and bar charts were presented for qualitative variables
- Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of P16 and TOP2A expression were calculated with cervical lesions as the gold standard, using a 2x2 contingency table
- Post-stratification Chi-square or Fisher's exact test was applied to determine the association between qualitative variables (clinicopathological parameters)
- A p-value of less than 0.05 was considered significant

CHAPTER 4

RESULTS

This study was conducted at PNS Shifa hospital, Karachi and Dr. Ruth K. M. Pfau, Civil Hospital, Karachi from November 2023 to June 2024. A total of 60 Liquid-Based Cytology samples from high-risk patients were collected according to the inclusion criteria specified earlier and the sample size calculation. The related clinical history and associated risk factors were also obtained using the designed Performa and recorded data entered into the electronic database. Following data analysis, baseline characteristics, demographics features, risk factors and different clinicopathological parameters of the patients were subsequently compared in relation to cytological diagnosis, and expression level of P16 and TOP2A immunohistochemical markers.

4.1 Demographic profile of study population

The study included a total of 60 female patients, the mean age was 46.23±11.70 years. Majority of the patients, 55%, were ≤45 years and rest were more than 45 years. Furthermore, 21 (35%) were postmenopausal while 39 (65%) were premenopausal women. The majority of patients (31.7%) belong to Urdu speaking ethnicity followed by Pathans (23.3%). It was found that about 41.7% of patients received no formal education, whereas only 18.3% had completed elementary school, 13.3% had completed matric, and 8.3% had completed intermediate education. Merely 18% patients had graduation or higher degrees. Out of total 60 patients, 80% were housewives and 20% were working women. Majority (88.3%) of the women were married while rest were divorced or separated. Among 60 female participants, 8.3% had been married since less than 10 years, 31.7% since 11 to 20 years and about 60% had been married for more than 20 years. The majority of females (71.7%) were married between the ages of 15 and 20, 21.7% between the ages of 21 and 25, and only 6.7% participants married after 25 years Table 4.1 present the study population's detailed demographic profile, respectively.

Table 4.1 Descriptive statistics of demographic profile (n=60)

	Frequency	Percent (%)
Age (years); mean± std. dev	**46.23±11.70**	
Age Group		
≤45 years	33	55
>45 years	27	45
Menopausal status		
Pre-menopause	39	65
Post- menopause	21	35
Ethnicity		
Urdu speaking	19	31.7
Sindhi	9	15.0
Punjabi	7	11.7
Pathan	14	23.3
Baloch	3	5.0
Others	8	13.3
Education		
No formal education	25	41.7
Elementary	11	18.3
Matric	8	13.3
Inter	5	8.3
Graduate or above	11	18.3
Occupation		
House wife	48	80
Working women	12	20
Marital status		
Married	53	88.3
Divorced/Widowed	7	11.7
Marriage duration		
≤10 years	5	8.3
11-20 years	19	31.7
>20 years	36	60
Age at marriage		
15-20 years	43	71.7
21-25 years	13	21.7
>25 years	4	6.7

4.2 Menstrual health and hygiene practices

On average, the age at menarche among participants was 12.91±1.12 years. About 36.7% females had menarche ages <12 years while 63.3% had >12 years. About two-third (63.3%) of the females, the average menstrual cycle lasting between 4-7 days. Out of 60 females, 56.7% utilized reusable clothes while 43.3% used disposable pads. About 68.3% of females, the average number of menstrual hygiene products used in a day was less than 3 per day, while for rest of the females, it was more than 3 per day. Table 4.2 presents comprehensive descriptive statistics on menstrual health and hygiene practices.

Table 4.2 Frequency distribution of menarche and menstrual hygiene practices (n=60)

	Frequency	**Percent (%)**
Menarche age(years); mean± std. dev	**12.91±1.12**	
Menarche age group		
≤12 years	22	36.7
>12 years	38	63.3
Average menstrual cycle		
≤3 days	12	20
4-7 days	38	63.3
>7 days	10	16.7
Menstrual hygiene product		
Disposable pads	26	43.3
Re-useable cloths	34	56.7
Average use of menstrual hygiene products in a day		
<3 times	41	68.3
≥3 times	19	31.7
Menstrual hygiene product disposal routine		
Garbage bin	60	100

4.3 Risk factors of cervical cancer

The majority (63.3%) of the women had more than three children, with an average 6.73±4.12 children. Among 60 females, 5 (8.3%) of the females had positive smoking history while 91.6% were non-smokers. The majority 4 out of 5 women had a smoking history for more than 5 years. Average Number of cigarettes smoked per day was 4.6±2.6 cigarettes. Out of 60 females, 10% of women reported having multiple sexual partners (history of more than one marriage), and 18.3% of females had husbands having multiple sexual partners (polygamous marriage). History of urogenital tract infection was discovered in 30 (50%) of the cases. Moreover, 60% of the 30 females with positive history of urogenital tract infection had cervicitis, 23.3% had recurrent UTIs, whereas 16.7% had both. Out of total 60 patients, only 23 had a positive history of using contraceptives, 13% used the barrier contraceptive technique, 26.1% used injectables, 34.8 % used IUDs, and 26.1 % used OCPS. The average time spent using contraceptives was 8.43±7.92 years. Tables 4.3 includes comprehensive descriptive statistics on risk factors of cervical cancer.

Table 4.3 Frequency distribution of risk factor of cervical cancer (n=60)

	Frequency	**Percent (%)**
No. of children's; mean± std. dev	6.73±4.12	
No. of children's group		
None	5	8.3
1-3 children's	17	28.3
>3 children's	38	63.3
Smoking status		
Active smoker	5	8.3
Non-smoker	55	91.6
Smoking duration (n=5)		
<1 year	0	0
1-5 years	1	20
>5 years	4	80
No. of cigarettes smoked (per day)	4.6±2.6	
Multiple sexual partners		

Yes	6	10
No	54	90
Multiple sexual partners of husband		
Yes	11	18.3
No	49	81.7
History of previous urogenital tract infection		
Yes	30	50
No	30	50
Urogenital tract infection type (n=30)		
Cervicitis	18	60
Recurrent UTI	7	23.3
Recurrent UTI and cervicitis	5	16.7
Prolong use of oral or any other form of contraceptive's history		
Yes	23	38.3
No	37	61.7
Method of contraceptives (n=23)		
Barrier method	3	13
Injectable	6	26.1
IUD	8	34.8
OCPs	6	26.1
Contraceptives method duration (years)	8.43±7.92	

4.4 Cervical cancer screening and HPV vaccination history

Out of 60 females, only 9 had underwent screening for cervical cancer previously, but none had a positive history of prior HPV vaccination. Pap smear (15%) was the previous screening method. 1/7 patients had HSIL on prior Pap smear, 1/7 had inadequate on prior smear, 5/7 patients had normal prior Pap smear while 2/7 did not know the results. Table 4.4 provide comprehensive descriptive statistics on HPV vaccination history, and cervical cancer screening status among patients.

Table 4.4 Frequency distribution of cervical health screening and HPV vaccination history (n=60)

	Frequency	**Percent (%)**
Prior HPV vaccination history		
Yes	0	0
No	60	100
Prior screening for cervical cancer		
Yes	9	15
No	51	85
Method of prior screening		
Pap Smear	9	15
Never done	51	85
Prior screening result (n=9)		
Inadequate Sample	1	11.1
Normal	5	55.5
HSIL	1	11.1
Not Known	2	22.2

4.5 Gynaecological Symptoms

Among 60 female participants, 25% had intermenstrual bleeding, 20% had postcoital bleeding, 38.3% had dyspareunia, 56.7% had abnormal vaginal discharge, and 80% had lower abdomen pain while post-menopausal bleeding was reported by 15 out of the 21 post-menopausal women. Table 4.5 (Figure 4.1) provide comprehensive descriptive statistics on gynaecological symptoms present.

Table 4.5 Frequency distribution of gynaecological symptoms (n=60)

	Frequency	**Percent (%)**
Intermenstrual bleeding		
Yes	15	25
No	45	75
Postcoital bleeding		
Yes	12	20
No	48	80
Dyspareunia		
Yes	23	38.3
No	37	61.7
Abnormal vaginal discharge		
Yes	34	56.7
No	26	43.3
Lower abdominal pain		
Yes	48	80
No	12	20
Post-menopausal bleeding (n=21)		
Yes	15	71.4
No	6	28.6

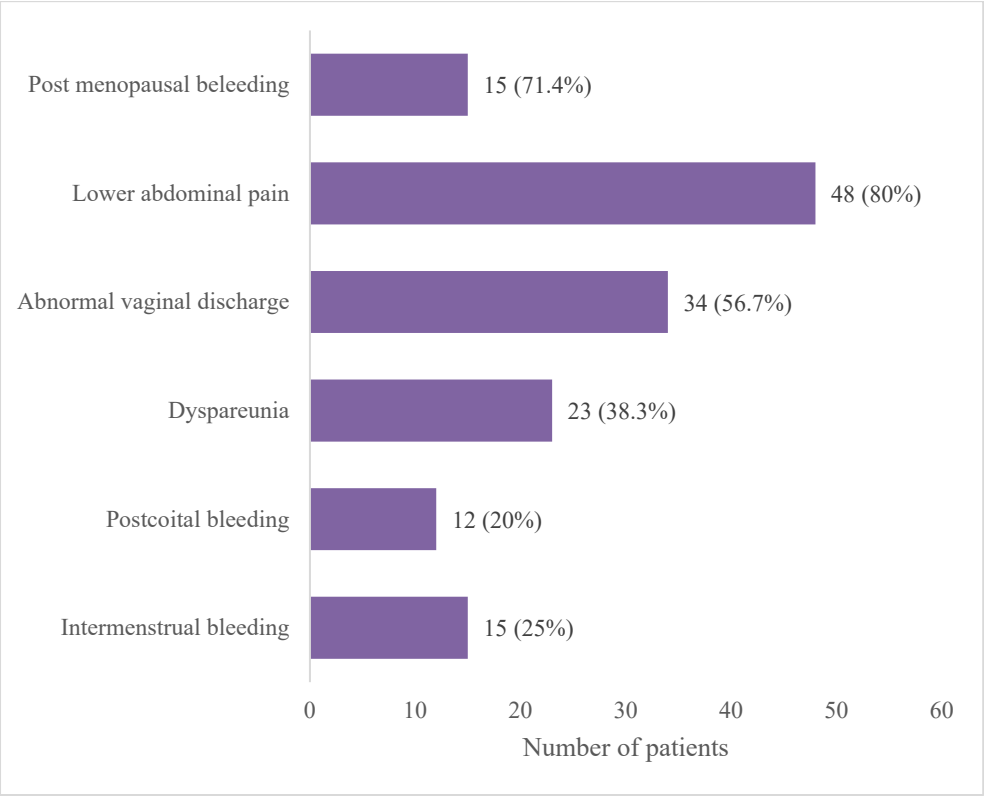


Figure 4.1 Bar chart presenting distribution of gynecological symptoms

4.6 Biopsy diagnosis, FIGO staging, Cancer status and Cytological diagnosis

Out of the total 12 biopsy confirmed cases, 9 had cancerous lesion, squamous cell carcinoma accounted for 41.7% whereas adenocarcinoma accounted for 25%, Poorly Differentiated accounted for 8.3% while 25% of patients had benign lesion. Patients were classified as having FIGO stage I-B (22.2%), stage II-A (22.2%), stage II-B (22.2%), and stage III-A (33.3%), respectively. Out of 60 patients, 55% had non-cancerous, 41.7% had pre-cancerous lesion, and 3.3% had cancerous lesion on cytology. Mean age of patients was 41.93±8.66 years, 51.72±13.27 years, and 48.50±9.19 years for non- cancerous, pre-cancerous, and cancerous patients respectively. The most frequent cytological diagnoses were NILM (26.7%), inflammatory (28.3%), and ASC-US (16.7%). Tables 4.6 (Figure 4.2 to Figure 4.5) provide comprehensive descriptive statistics for the cytological diagnosis, FIGO stage, cancer status, and biopsy diagnosis.

Table 4.6 Frequency distribution of Biopsy diagnosis, FIGO staging, Cancer status and Cytological diagnosis

	Frequency	**Percent (%)**
Biopsy diagnosis (n=-12)		
Adenocarcinoma	3	25
Squamous cell carcinoma	5	41.7
Poorly differentiated	1	8.3
Benign	3	25
FIGO Stage (n=9)		
Stage I-B	2	22.2
Stage II-A	2	22.2
Stage II-B	2	22.2
Stage III-A	3	33.3
Cancer status		
Non-cancerous	33	55
Pre-Cancerous	25	41.7
Cancerous	2	3.3
Cytological diagnosis		
NILM	16	26.7
Inflammatory	17	28.3
ASC-US	10	16.7
Atypical glandular cells	4	6.7
LSIL	5	8.3
HSIL	6	10
Invasive carcinoma	2	3.3

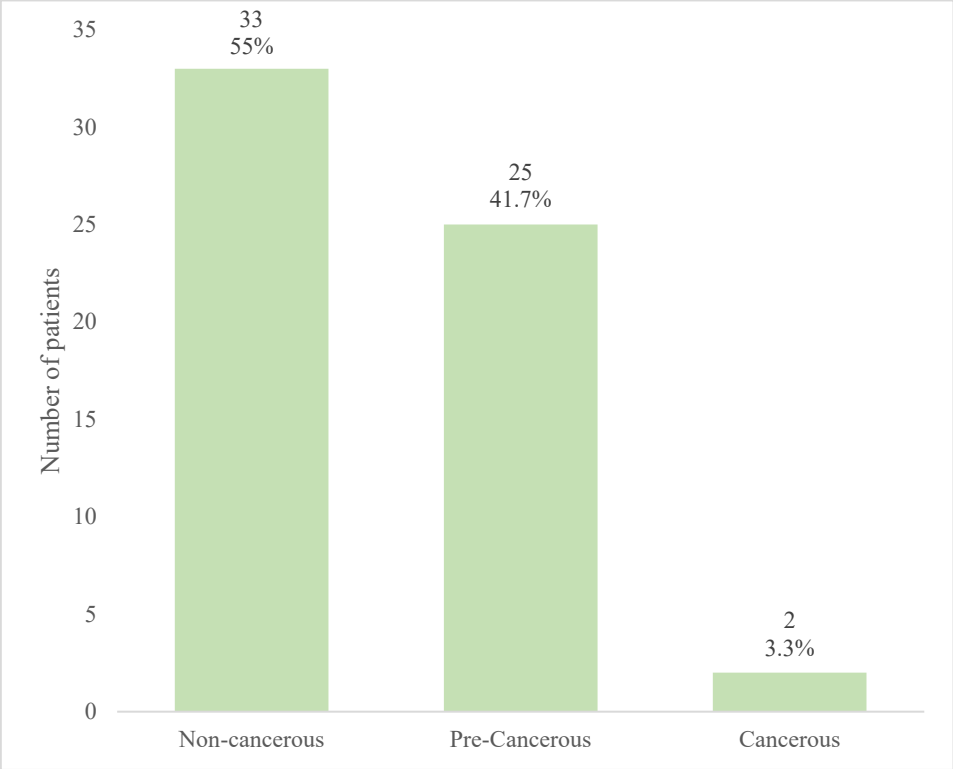


Figure 4.2 Bar chart presenting distribution of cancer status

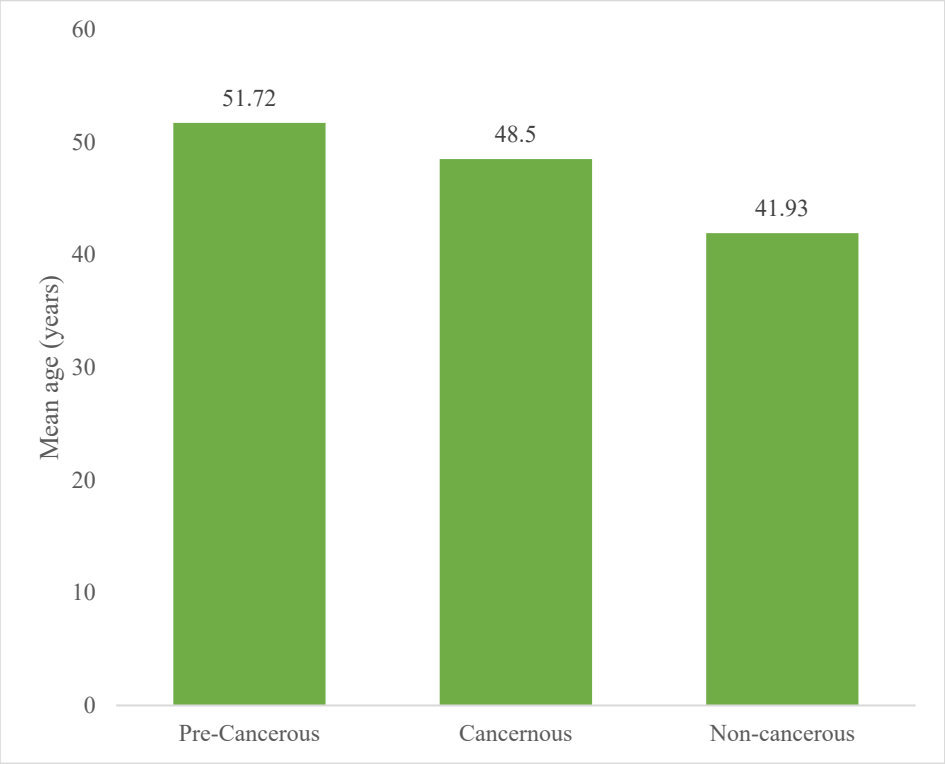


Figure 4.3 Mean age of patients according to cancer status

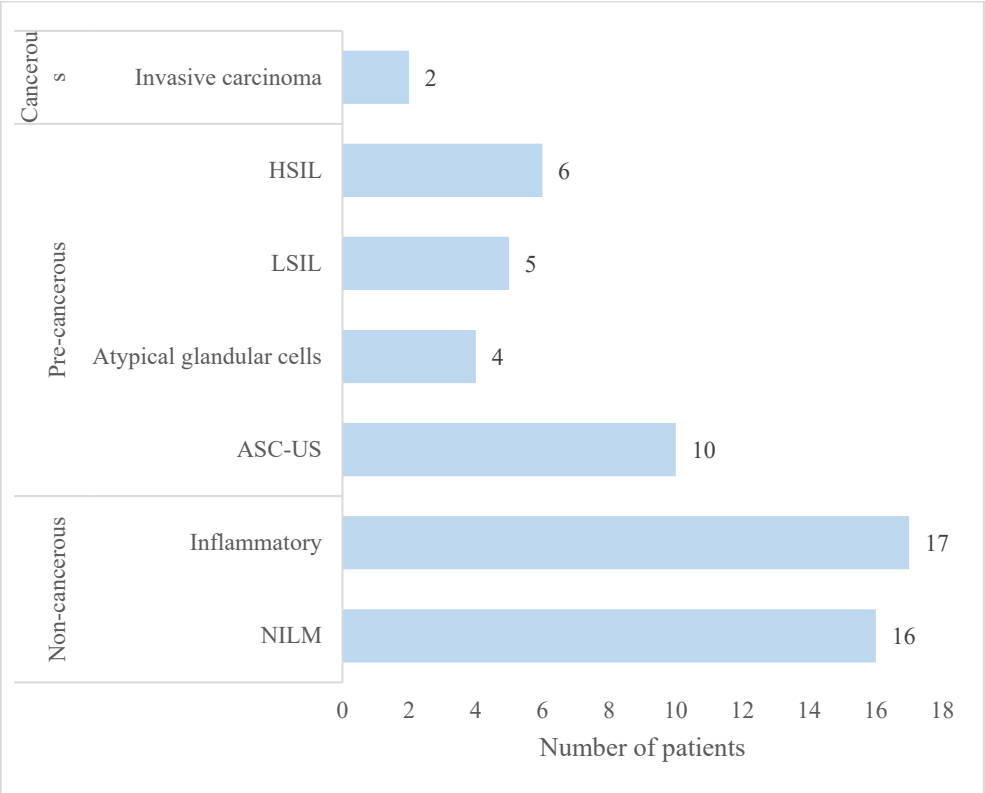


Figure 4.4 Bar chart presenting distribution of cytological diagnosis

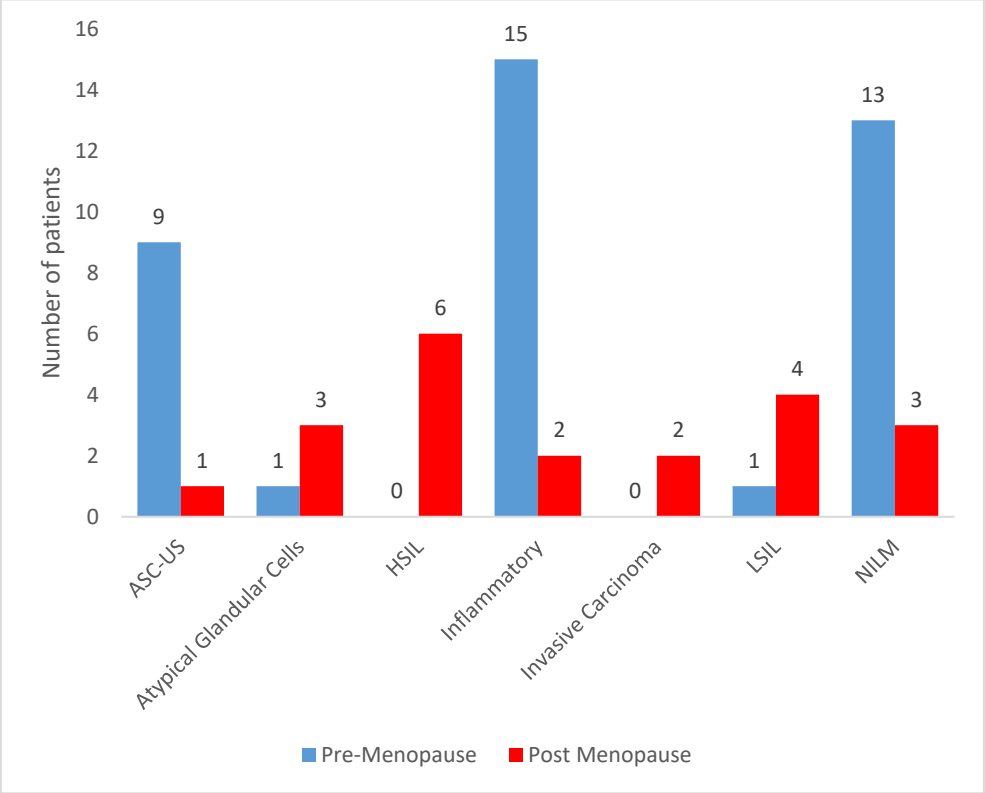


Figure 4.5 Bar chart presenting distribution of cytological diagnosis according to menopausal status

4.7 Cervical lesion, P16 and TOP2A percentage of positive cells, staining intensity and expression

The cytology samples were subjected to IHC using anti-P16 and anti-TOP2A antibodies and staining was analyzed for both proportion along with intensity of the cells.

Out of total 60 cases, it was observed that 22(36.7%) of patients were positive for P16 expression, whereas only 6(10%) were positive for TOP2A expression. Out of 22 cases with positive staining for P16, 81.8% showed positive staining in 10–50% of cells, 4.5% showed positive staining in 51–80% of cells, while only 13.6% showed positive staining in >80% of cells. Out of 6 cases with positive TOP2A expression, only 16.7% showed positive staining in <10% of cells, while 83.3% showed positive staining in 10–50% of cells. In 22 cases with positive P16 expression, 36.4% had mild intensity staining, 36.4% had moderate intensity staining, while only 27.2% had strong intensity staining. In 6 cases with positive TOP2A staining, 50% had mild intensity staining, 33.3% had moderate intensity staining, and only 16.7% had strong intensity staining. Based on IRS-scoring 36.4% had mild, 50% had moderate, 13.6% had severe P16 expression levels, while 66.7% had mild, 33.3% had moderate TOP2A expression levels. Further 27(45%) patients showed pre-cancerous and cancerous cervical lesion while the rest show normal cervical cytology. Table 4.7 and Table-8 (Figure 4.6 through Figure 4.8) provide comprehensive descriptive statistics.

Table 4.7 Frequency distribution of P16 and TOP2A Proportion and staining intensity

	Frequency	**Percent (%)**
P16 Proportion (n=22)		
<10% of positive cells	0	0
10-50% positive cells	18	81.8
51-80% of positive cells	1	4.5
>80% positive cells	3	13.6
P16 staining intensity (n=22)		
Mild reaction	8	36.4
Moderate Reaction	8	36.4
Strong reaction	6	27.2
TOP2A Proportion (n=6)		
<10% of positive cells	1	16.7
10-50% positive cells	5	83.3
51-80% of positive cells	0	0
>80% positive cells	0	0
TOP2A staining intensity (n=6)		
Mild reaction	3	50
Moderate Reaction	2	33.3
Strong reaction	1	16.7

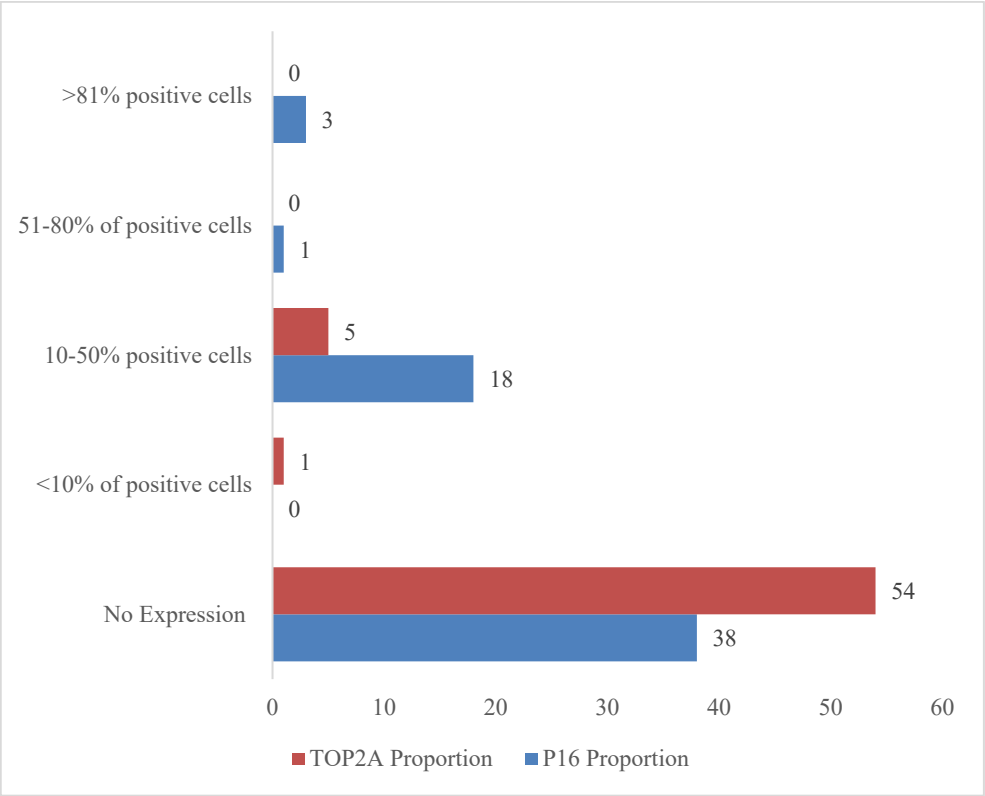


Figure 4.6 Bar chart presenting distribution of P16 and TOP2A proportion

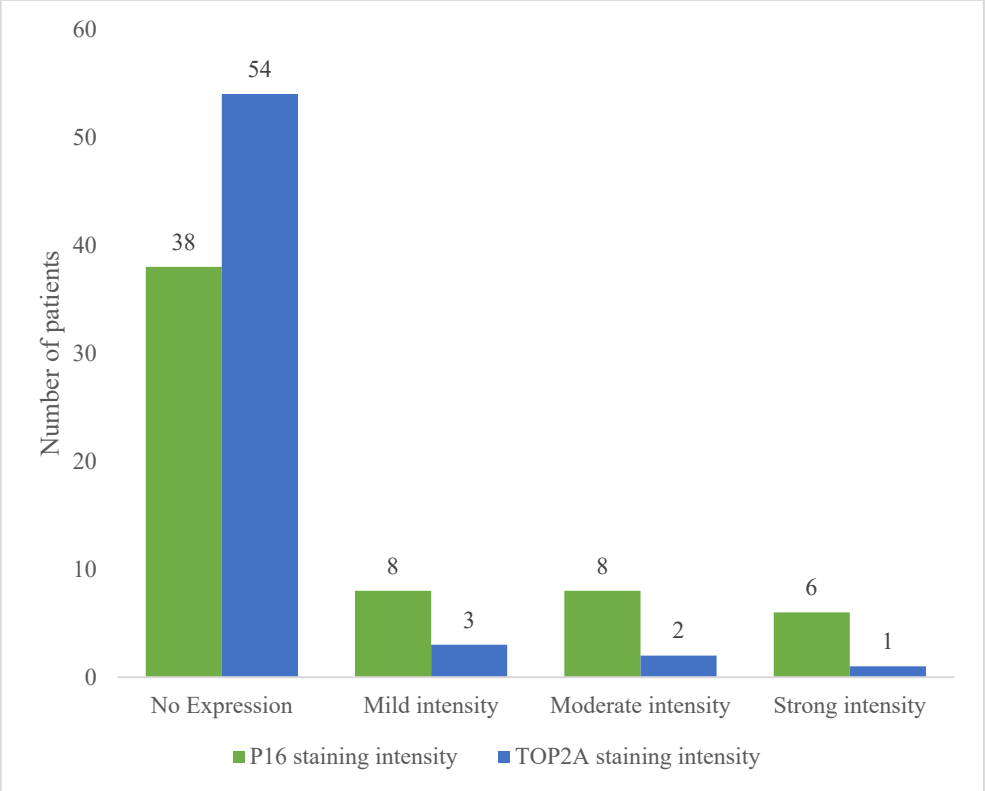


Figure 4.7 Bar chart presenting distribution of P16 and TOP2A staining intensity

Table 4.8 Frequency distribution of P16 and TOP2A expression Based on IRS- score (n=60)

	Frequency	Percent (%)
P16 antibody expression		
Positive	22	36.7
Negative	38	63.3
P16 antibody expression severity (n=22)		
Mild	8	36.4
Moderate	11	50.0
Severe	3	13.6
TOP2A antibody expression		
Positive	6	10
Negative	54	90
TOP2A antibody expression severity (n=6)		
Mild	4	66.7
Moderate	2	33.3
Cervical lesion		
Positive	27	45
Negative	33	55

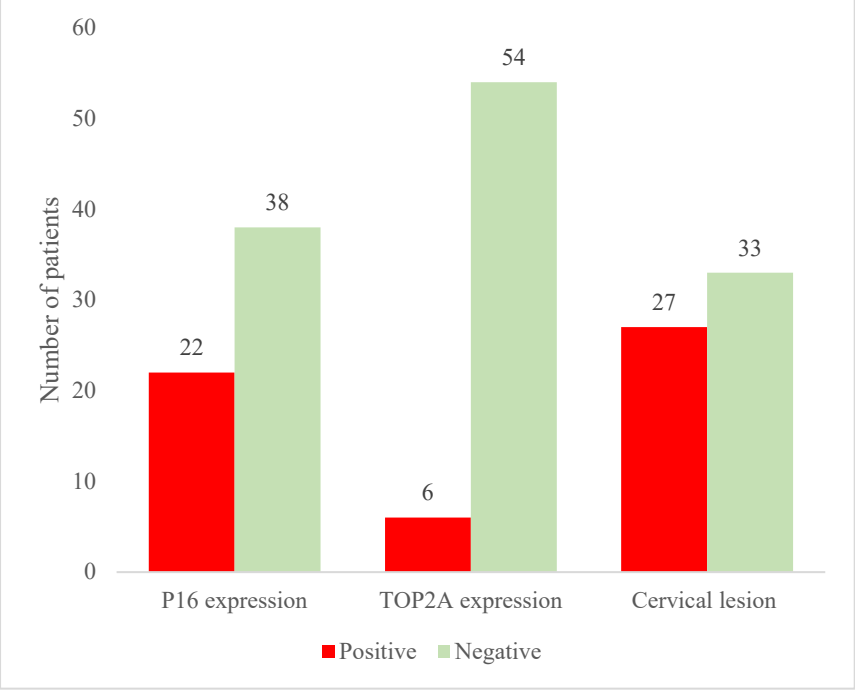


Figure 4.8 Bar chart presenting distribution of expression of P16, TOP2A and cervical lesion

4.8 Diagnostic accuracy of P16 and TOP2A expression in early detection of cervical cancer

The sensitivity, specificity, predictive values, and diagnostic accuracy were determined for P16 expression and TOP2A expression using the cervical lesion as the gold standard for the early detection of cervical cancer. For the P16 expression, 20 patients were true positive, correctly diagnosed as positive and 31 patients were true negative, correctly diagnosed as negative. Sensitivity, Specificity, PPV, NPV and accuracy were 74.1%, 93.9%, 90.9%, 81.6% and 85% respectively.

For the TOP2A expression, 6 patients were true positive, correctly diagnosed as positive and 33 patients were true negative, correctly diagnosed as negative. Sensitivity, Specificity, PPV, NPV and accuracy were 22.2%, 100%, 100%, 61.1% and 65% respectively. Detailed results are presented in Table 4.9 (Figure 4.9 to Figure 4.12).

Table 4.9 Diagnostic accuracy of P16 and TOP2A in detection of cervical cancer with cervical lesion as gold standard (n=60)

		Cervical lesion Frequency			**P-VALUE**
		Positive	**Negative**	**Total**	
P16 expression	**Positive**	20	2	**22**	**0.000***
	Negative	7	31	**38**	
	Total	**27**	**33**	**60**	
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
	74.1%	**93.9%**	**90.9%**	**81.6%**	**85%**
TOP2A expression	**Positive**	**6**	**0**	**6**	**0.006**
	Negative	**21**	**33**	**54**	
	Total	**27**	**33**	**60**	
	Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
	22.2%	**100%**	**100%**	**61.1%**	**65%**

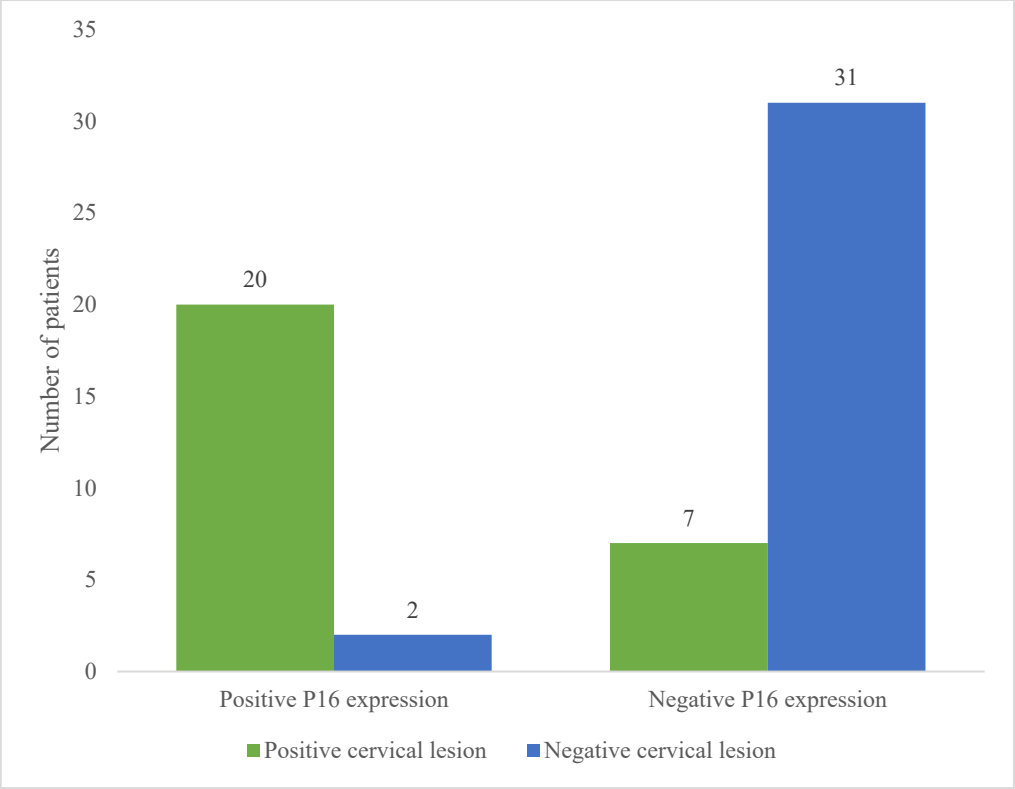


Figure 4.9 Bar chart presenting distribution of expression of P16 expression according to cervical lesion

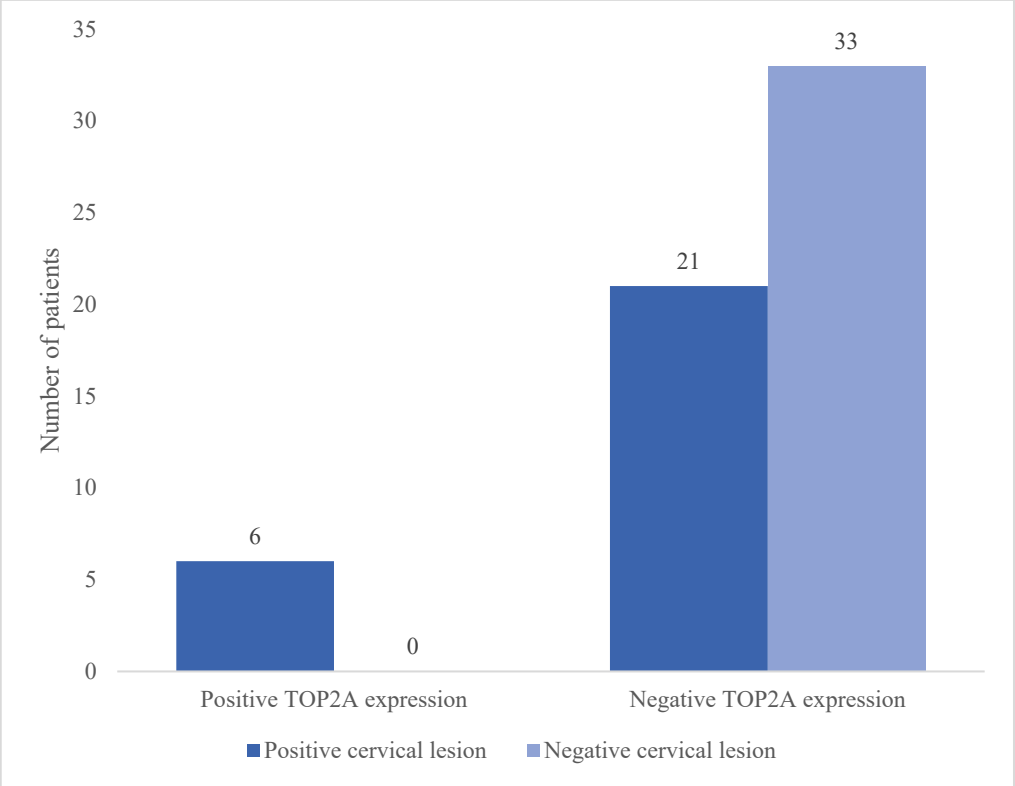


Figure 4.10 Bar chart presenting distribution of expression of TOP2A expression according to cervical lesion

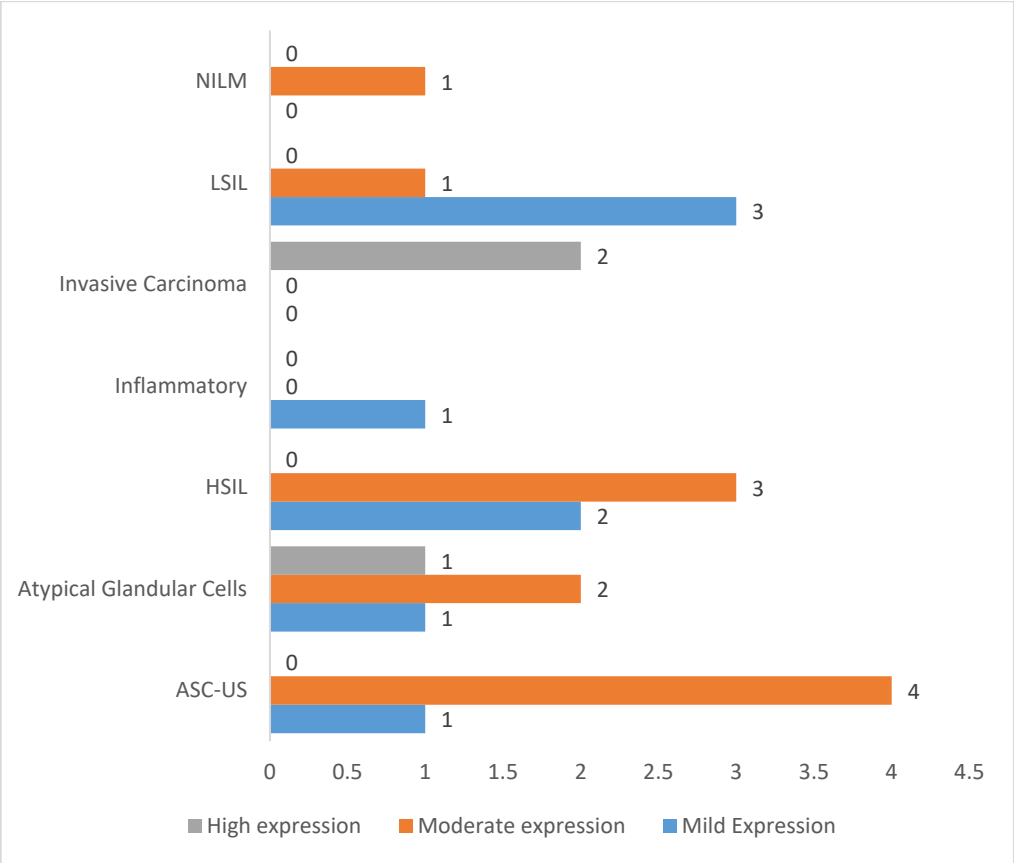


Figure 4.11 P16 severity according to cytological diagnosis

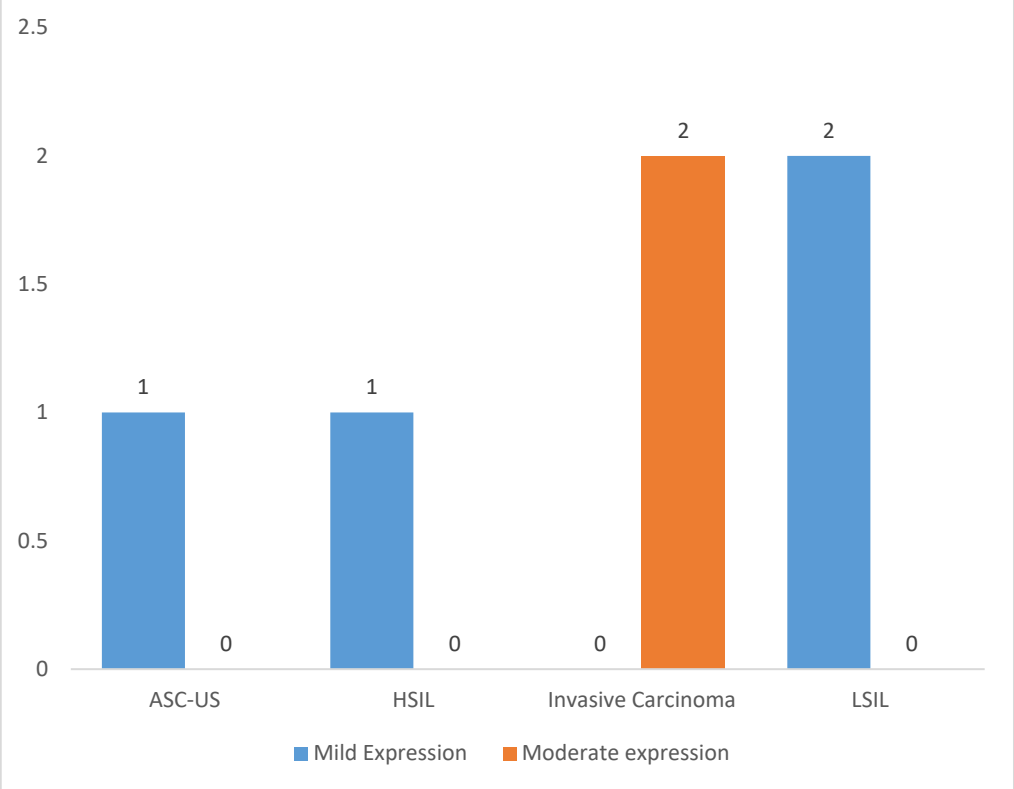


Figure 4.12 TOP2A severity according to cytological diagnosis

4.9 Association of P16 and TOP2A expression with risk factors

We evaluated the association of P16 and TOP2A expression with various parameters including risk factors. A significant association of P16 expression with age group (p=0.027), ethnicity (p=0.020), menopausal status (p=0.000), cytological diagnosis (p=0.000) and cancer status (p=0.000) was found. Moreover, a significant association of TOP2A expression with menopausal status (p=0.017), cytological diagnosis (p=0.000) and cancer status (p=0.000) was noted. Detailed results of association are presented from Table 4.10 to Table 4.21 respectively

Table 4.10 Frequency and association of P16 anti-body expression with demographic characteristics (n=60)

		P16 anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Age Group	≤45 years	8(36.4)	25(65.8)	0.027*
	>45 years	14(63.6)	13(34.2)	
Menopausal status	Pre-menopause	8(36.4)	31(81.6)	0.000*
	Post-menopause	14(63.6)	7(18.4)	
Marital Status	Married	18(81.8)	35(92.1)	0.405
	Divorced/widowed	4(18.2)	3(7.9)	
Marriage duration	≤10 years	0(0)	5(13.2)	0.078
	11-20 years	5(22.7)	14(36.8)	
	>20 years	17(77.3)	19(50)	
Ethnicity	Urdu Speaking	11(50)	8(21.1)	0.020*
	Sindhi	1(4.5)	8(21.1)	
	Punjabi	0(0)	7(18.4)	
	Pathan	4(18.2)	10(26.3)	
	Baloch	2(9.1)	1(2.6)	
	Others	4(18.2)	4(10.5)	
Education	No Formal Education	9(40.9)	16(42.1)	0.819
	Elementary	3(13.6)	8(21.1)	
	Matric	3(13.6)	5(13.2)	

	Inter	3(13.6)	2(5.3.)	
	Graduate or above	4(18.2)	7(18.4)	
Occupation	House wife	16(72.7)	32(84.2)	0.327
	Working Women	6(27.3)	6(15.8)	
Age at marriage	15-20 years	16(72.7)	27(71.1)	1.000
	21-25 years	5(22.7)	8(21.1)	
	>25 years	1(4.5)	3(7.9)	
Number of children's	None	0(0)	5(13.2)	0.148
	1-3 children's	5(22.7)	12(31.6)	
	>3 children's	17(77.3)	21(55.3)	

Table 4.11 Frequency and association of P16 anti-body expression with smoking history, status, and duration (n=60)

		P16 anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Smoking status	Active smoker	3(13.6)	2(5.3)	0.346
	Non-smoker	19(86.4)	36(94.7)	
Smoking duration (n=5)	<1 year	0(0)	0(0)	1.000
	1-5 years	1(33.3)	0(0)	
	>5 years	2(66.7)	2(100)	

Table 4.12 Frequency and association of P16 anti-body expression with multiple sexual partnerships among women and their husbands (n=60)

		P16 anti-body expression n (%)		p-value
		Positive	**Negative**	
Multiple sexual partners	Yes	2(9.1)	4(10.5)	1.000
	No	20(90.9)	34(89.5)	
Multiple sexual partners of husband	Yes	3(13.6)	8(21.1)	0.731
	No	19(86.4)	30(78.9)	

Table 4.13 Frequency and association of P16 anti-body expression with UTI history, contraceptive use, and cervical cancer screening (n=60)

		P16 anti-body expression n (%)		p-value
		Positive	**Negative**	
History of previous Urogenital Tract Infection	Yes	12(54.5)	18(47.4)	0.606
	No	10(45.5)	20(52.6)	
Prolong use of oral or other form of contraceptive's history	Yes	9(40.9)	14(36.8)	0.755
	No	13(59.1)	24(63.2)	
Method of contraceptives (n=23)	Barrier method	2(22.2)	1(7.1)	0.235
	Injectable	1(11.1)	5(35.7)	
	IUD	2(22.2)	6(42.9)	
	OCPS	4(44.4)	2(14.3)	
Prior screening for cervical cancer	Yes	3(13.6)	5(13.2)	1.000
	No	19(86.4)	33(86.8)	

Table 4.14 Frequency and association of P16 anti-body expression with gynecological symptoms (n=60)

		P16 anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Inter-menstrual bleeding	Yes	4(18.2)	11(28.9)	0.353
	No	18(81.8)	27(71.1)	
Postcoital bleeding	Yes	3(13.6)	9(23.7)	0.507
	No	19(86.4)	29(76.3)	
Dyspareunia	Yes	6(27.3)	17(44.7)	0.180
	No	16(72.7)	21(55.3)	
Abnormal vaginal discharge	Yes	13(59.1)	21(55.3)	0.773
	No	9(40.9)	17(44.7)	
Lower abdominal pain	Yes	20(90.9)	28(73.7)	0.180
	No	2(9.1)	10(26.3)	
Post menstrual bleeding	Yes	12(85.7)	3(42.9)	0.120
	No	2(14.3)	4(57.1)	

Table 4.15 Frequency and association of P16 anti-body expression with biopsy diagnosis, FIGO stage, cancer status, cytological diagnosis (n=60)

		P16 anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Biopsy Diagnosis (n=12)	Adenocarcinoma	3(30)	0(0)	0.258
	Squamous Cell Carcinoma	4(40)	1(50)	
	Poorly differentiated carcinoma	0(0)	1(50)	
	Benign	3(30)	0(0)	
FIGO Stage (n=9)	Stage I-B	2(28.6)	0(0)	1.000
	Stage II-A	1(14.3)	1(50)	
	Stage II-B	2(28.6)	0(0)	
	Stage III-A	2(28.6)	1(50)	
Cancer status	Pre-cancerous	18(81.8)	7(18.4)	0.000*
	Cancerous	2(9.1)	0(0)	
	Non- cancerous	2(9.1)	31(81.6)	
Cytological Diagnosis	NILM	1(4.5)	15(39.5)	0.000*
	Inflammatory	1(4.5)	16(42.1)	
	ASC-US	5(22.7)	5(13.2)	
	Atypical Glandular Cells	4(18.2)	0(0)	
	LSIL	4(18.2)	1(2.6)	
	HSIL	5(22.7)	1(2.6)	
	Invasive Carcinoma	2(9.1)	0(0)	

Table 4.16 Frequency and association of TOP2A anti-body expression with demographic characteristics (n=60)

		TOP2A anti-body expression n (%)		p-value
		Positive	**Negative**	
Age Group	≤45 years	2(33.3)	31(57.4)	0.394
	>45 years	4(66.7)	23(42.6)	
Menopausal status	Pre-menopause	1(16.7)	38(70.4)	0.017*
	Post-menopause	5(83.3)	16(29.6)	
Marital Status	Married	6(100)	47(87)	1.000
	Divorced/widowed	0(0)	7(13)	
Marriage duration	≤10 years	0(0)	5(9.3)	0.799
	11-20 years	1(16.7)	18(33.3)	
	>20 years	5(83.3)	31(57.4)	
Ethnicity	Urdu Speaking	3(50)	16(29.6)	0.436
	Sindhi	0(0)	9(16.7)	
	Punjabi	0(0)	7(13)	
	Pathan	1(16.7)	13(24.1)	
	Baloch	1(16.7)	2(3.7)	
	Others	1(16.7)	7(13)	
Education	No Formal Education	2(33.3)	23(42.6)	0.471
	Elementary	0(0)	11(20.4)	
	Matric	1(16.7)	7(13)	
	Inter	1(16.7)	4(7.4)	
	Graduate or above	2(33.3)	9(16.7)	
Occupation	House wife	5(83.3)	43(79.6)	1.000
	Working Women	1(16.7)	11(20.4)	
Age at marriage	15-20 years	4(66.7)	39(72.2)	0.558
	21-25 years	1(16.7)	12(22.2)	
	>25 years	1(16.7)	3(5.6)	
Number of children's	None	0(0)	5(9.3)	0.799
	1-3 children's	1(16.7)	16(29.6)	
	>3 children's	5(83.3)	33(61.1)	

Table 4.17 Frequency and association of TOP2A anti-body expression with smoking history, status, and duration (n=60)

		TOP2A anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Smoking status	Active smoker	2(33.3)	3(5.6)	0.074
	Non-smoker	4(66.7)	51(94.4)	
Smoking duration (n=5)	<1 year	0(0)	0(0)	0.400
	1-5 years	1(50)	0(0)	
	>5 years	1(50)	3(100)	

Table 4.18 Frequency and association of TOP2A anti-body expression with multiple sexual partnerships among women and their husbands (n=60)

		TOP2A anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Multiple sexual partners	Yes	0(0)	6(11.1)	1.000
	No	6(100)	48(88.9)	
Multiple sexual partners of husband	Yes	1(16.7)	10(18.5)	1.000
	No	5(83.3)	44(81.5)	

Table 4.19 Frequency and association of TOP2A anti-body expression with medical history, contraceptive use, and cervical cancer screening (n=60)

		TOP2A anti-body expression n (%)		**p-value**
		Positive	**Negative**	
History of previous Urogenital Tract Infection	Yes	4(66.7)	26(48.1)	0.434
	No	2(33.3)	28(51.9)	
Prolong use of oral or other form of contraceptives history	Yes	3(50)	20(37)	0.666
	No	3(50)	34(63)	
Method of contraceptives (n=23)	Barrier method	1(33.3)	2(10)	0.108
	Injectable	0(0)	6(30)	
	IUD	0(0)	8(40)	
	OCPS	2(66.7)	4(20)	
Prior screening for cervical cancer	Yes	1(16.7)	7(13)	1.000
	No	5(83.3)	47(87)	

Table 4.20 Frequency and association of TOP2A anti-body expression with symptoms (n=60)

		TOP2A anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Inter-menstrual bleeding	Yes	1(16.7)	14(25.9)	1.000
	No	5(83.3)	40(74.1)	
Postcoital bleeding	Yes	0(0)	12(22.2)	0.333
	No	6(100)	42(77.8)	
Dyspareunia	Yes	1(16.7)	22(40.7)	0.391
	No	5(83.3)	32(59.3)	
Abnormal vaginal discharge	Yes	3(50)	31(57.4)	1.000
	No	3(50)	23(42.6)	
Lower abdominal pain	Yes	5(83.3)	43(79.6)	1.000
	No	1(16.7)	11(20.4)	
Post menstrual bleeding	Yes	4(80)	11(68.8)	1.000
	No	1(20)	5(31.3)	

Table 4.21 Frequency and association of TOP2A anti-body expression with biopsy diagnosis, FIGO stage, cancer status and cytological diagnosis (n=60)

		TOP2A anti-body expression n (%)		**p-value**
		Positive	**Negative**	
Biopsy diagnosis (n=12)	Adenocarcinoma	0(0)	3(30)	0.545
	Squamous cell carcinoma	2(100)	3(30)	
	Poorly differentiated carcinoma	0(0)	1(10)	
	Benign	0(0)	3(30)	
FIGO stage (n=9)	Stage I-B	0(0)	2(28.6)	1.000
	Stage II-A	0(0)	2(28.6)	
	Stage II-B	1(50)	1(14.3)	
	Stage III-A	1(50)	2(28.6)	
Cancer Status	Pre-cancerous	4(66.7)	21(38.9)	0.000*
	cancerous	2(33.3)	0(0)	
	Non- cancerous	0(0)	33(61.1)	
Cytological diagnosis	NILM	0(0)	16(29.6)	0.000*
	Inflammatory	0(0)	17(31.5)	
	ASC-US	1(16.7)	9(16.7)	
	Atypical glandular cells	0(0)	4(7.4)	
	LSIL	2(33.3)	3(5.6)	
	HSIL	1(16.7)	5(9.3)	
	Invasive carcinoma	2(33.3)	0(0)	

4.10 Association of Cytological Diagnosis with Risk Factors

We evaluated the association of cytological diagnosis with various parameters including risk factors. A significant association with age group (p=0.000), menopausal status (p=0.000), smoking status (p=0.000), biopsy diagnosis (p=0.003) and dyspareunia (p=0.011) was found. Detailed results of association are presented in Table 4.22 and Table 4.23 respectively.

Figures 4.13 to 4.24 display the cytological evaluation, and immunohistochemical expression of P16 and TOP2A in 3 cancerous cases included in this study.

Table 4.22 Frequency and association of cytological diagnosis with various risk factors (n=60)

		Cytological diagnosis n (%)							**p-value**
		NILM	**Inflammatory**	**ASC-US**	**Atypical glandular cells**	**LSIL**	**HSIL**	**Invasive carcinoma**	
Age Group	≤45 years	11(68.8)	13(76.5)	8(80)	0(0)	0(0)	0(0)	1(50)	0.000*
	>45 years	5(31.3)	4(23.5)	2(20)	4(100)	5(100)	6(100)	1(50)	
Menopausal status	Pre-menopause	13(81.3)	15(88.2)	9(90)	1(25)	1(20)	0(0)	0(0)	0.000*
	Post-menopause	3(18.7)	2(11.2)	1(10)	3(75)	4(80)	6(100)	2(100)	
Marital Status	Married	15(93.8)	16(94.1)	9(90)	3(75)	4(80)	4(66.7)	2(100)	0.390
	Divorced/widowed	1(6.3)	1(5.9)	1(10)	1(25)	1(20)	2(33.3)	0(0)	
Marriage duration	≤10 years	3(18.8)	1(5.9)	1(10)	0(0)	0(0)	0(0)	0(0)	0.066
	11-20 years	5(31.3)	8(47.1)	6(60)	0(0)	0(0)	0(0)	0(0)	
	>20 years	8(50)	8(47.1)	3(30)	4(100)	5(100)	6(100)	2(100)	
Age at marriage	15-20 years	11(68.8)	13(76.5)	7(70)	2(50)	3(60)	5(83.3)	2(100)	0.861
	21-25 years	3(18.8)	4(23.5)	2(20)	2(50)	1(20)	1(16.7)	0(0)	
	>25 years	2(12.5)	0(0)	1(10)	0(0)	1(20)	0(0)	0(0)	
	None	2(12.5)	2(11.8)	1(10)	0(0)	0(0)	0(0)	0(0)	0.333

Number of children's	1-3 children's	5(31.3)	7(41.2)	1(10)	3(75)	1(20)	0(0)	0(0)	
	>3 children's	9(56.3)	8(47.1)	8(80)	1(25)	4(80)	6(100)	2(100)	
Smoking status	Active smoker	0(0)	1(5.9)	0(0)	0(0)	0(0)	2(33.3)	2(100)	0.000*
	Non-smoker	16(100)	16(94.1)	10(100)	4(100)	5(100)	4(66.7)	0(0)	
Multiple sexual partners	Yes	4(25)	1(5.9)	1(10)	0(0)	0(0)	0(0)	0(0)	0.592
	No	12(75)	16(94.1)	9(90)	4(100)	5(100)	6(100)	2(100)	
Multiple sexual partners of husband	Yes	5(31.3)	3(17.6)	1(10)	0(0)	1(20)	0(0)	1(50)	0.481
	No	11(68.8)	14(82.4)	9(90)	4(100)	4(80)	6(100)	1(50)	
History of previous Urogenital Tract Infection	Yes	7(43.8)	9(52.9)	7(70)	2(50)	3(60)	1(16.7)	1(50)	0.575
	No	9(56.3)	8(47.1)	3(30)	2(50)	2(40)	5(83.3)	1(50)	
Prolong use of oral or other form of contraceptive's history	Yes	7(43.8)	7(41.2)	4(40)	2(50)	1(20)	1(16.7)	1(50)	0.887
	No	9(56.3)	10(58.8)	6(60)	2(50)	4(80)	5(83.3)	1(50)	
	Yes	2(12.5)	1(5.9)	3(30)	1(25)	0(0)	0(0)	1(50)	0.228

Prior screening for cervical cancer	No	14(87.5)	16(94.1)	7(70)	3(75)	5(100)	6(100)	1(50)	
Screening Results	Normal	2(12.5)	1(5.9)	1(10)	1(25)	0(0)	0(0)	0(0)	0.269
	HSIL	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	
	Inadequate	0(0)	0(0)	1(10)	0(0)	0(0)	0(0)	0(0)	
	Not Known	14(87.5)	16(94.1)	8(80)	3(75)	5(100)	6(100)	1(50)	
Biopsy diagnosis (n=12)	Adenocarcinoma	0(0)	0(0)	0(0)	3(75)	0(0)	0(0)	0(0)	0.003*
	Squamous cell carcinoma	0(0)	0(0)	0(0)	0(0)	0(0)	3(100)	2(100)	
	Poorly differentiated carcinoma	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	
	Benign	0(0)	1(100)	0(0)	1(25)	1(100)	0(0)	0(0)	

Table 4.23 Frequency and association of cytological diagnosis with symptoms (n=60)

		Cytological diagnosis n (%)							p-value
		NILM	**Inflammatory**	**ASC-US**	**Atypical glandular cells**	**LSIL**	**HSIL**	**Invasive carcinoma**	
Inter-menstrual bleeding	Yes	3(18.8)	7(41.2)	3(30)	1(25)	0(0)	1(16.7)	0(0)	0.601
	No	13(81.3)	10(58.8)	7(70)	3(75)	5(100)	5(83.3)	2(100)	
Postcoital bleeding	Yes	6(37.5)	5(29.4)	1(10)	0(0)	0(0)	0(0)	0(0)	0.293
	No	10(62.5)	12(70.6)	9(90)	4(100)	5(100)	6(100)	2(100)	
Dyspareunia	Yes	7(43.8)	10(58.8)	6(60)	0(0)	0(0)	0(0)	0(0)	0.011*
	No	9(56.3)	7(41.2)	4(40)	4(100)	5(100)	6(100)	2(100)	
Abnormal vaginal discharge	Yes	9(56.3)	10(58.8)	7(70)	3(75)	2(40)	2(33.3)	1(50)	0.800
	No	7(43.8)	7(41.2)	3(30)	1(25)	3(60)	4(66.74)	1(50)	
Lower abdominal pain	Yes	10(62.5)	13(76.5)	10(100)	4(100)	3(60)	6(100)	2(100)	0.136
	No	6(37.5)	4(23.5)	0(0)	0(0)	2(40)	0(0)	0(0)	
Post menstrual bleeding	Yes	1(33.3)	1(50)	1(100)	2(66.7)	4(100)	5(83.3)	1(50)	0.466
	No	2(66.7)	1(50)	0(0)	1(33.3)	0(0)	1(16.7)	1(50)	

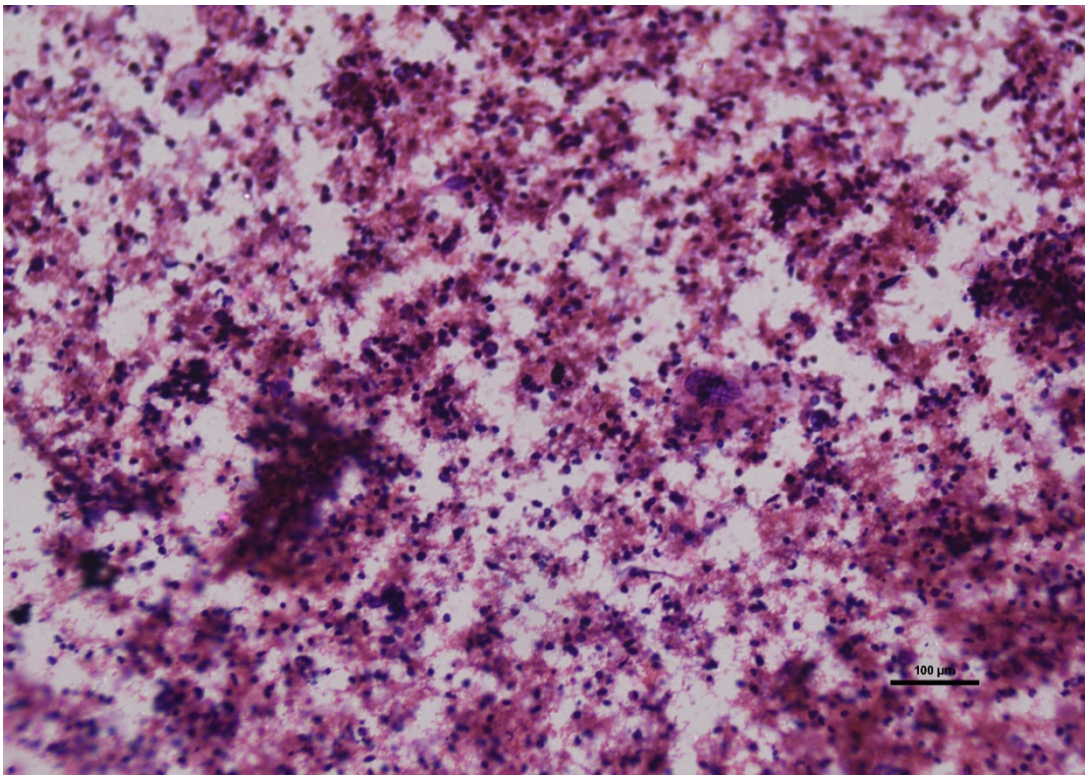


Figure 4.13 Invasive Cervical Carcinoma- of a 55-year-old female (PAP, x20)

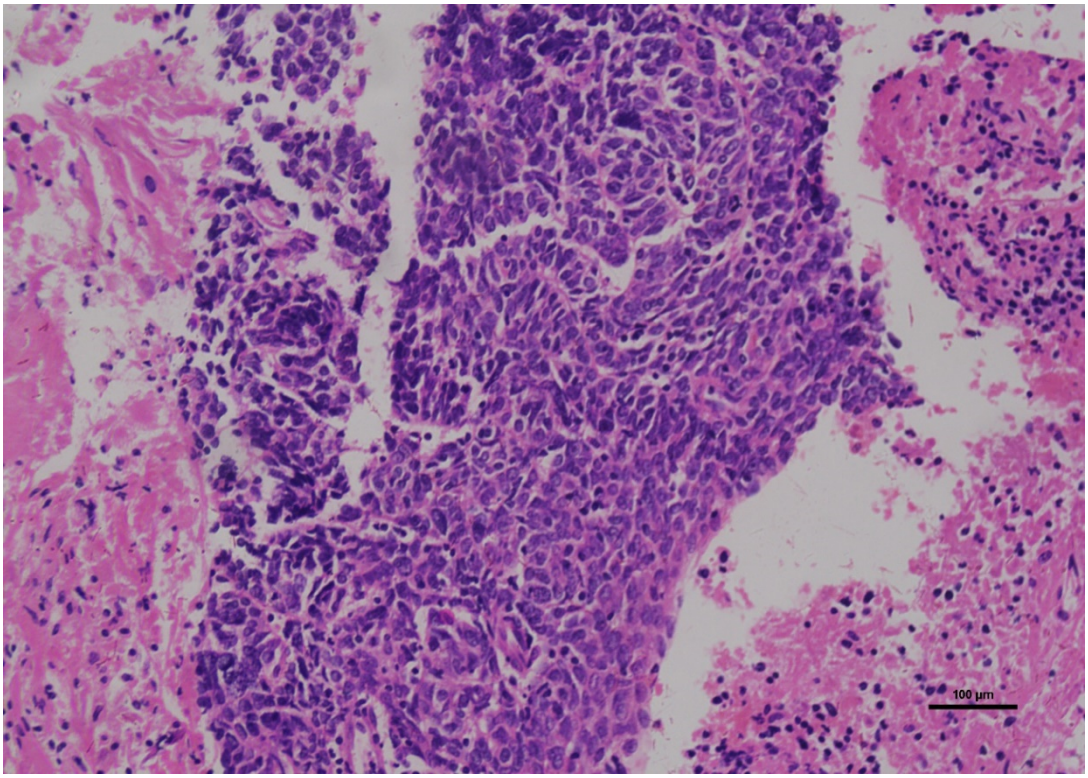


Figure 4.14 Invasive Cervical Carcinoma- of a 55-year-old female (H&E of same case as Figure 4.13, x20)

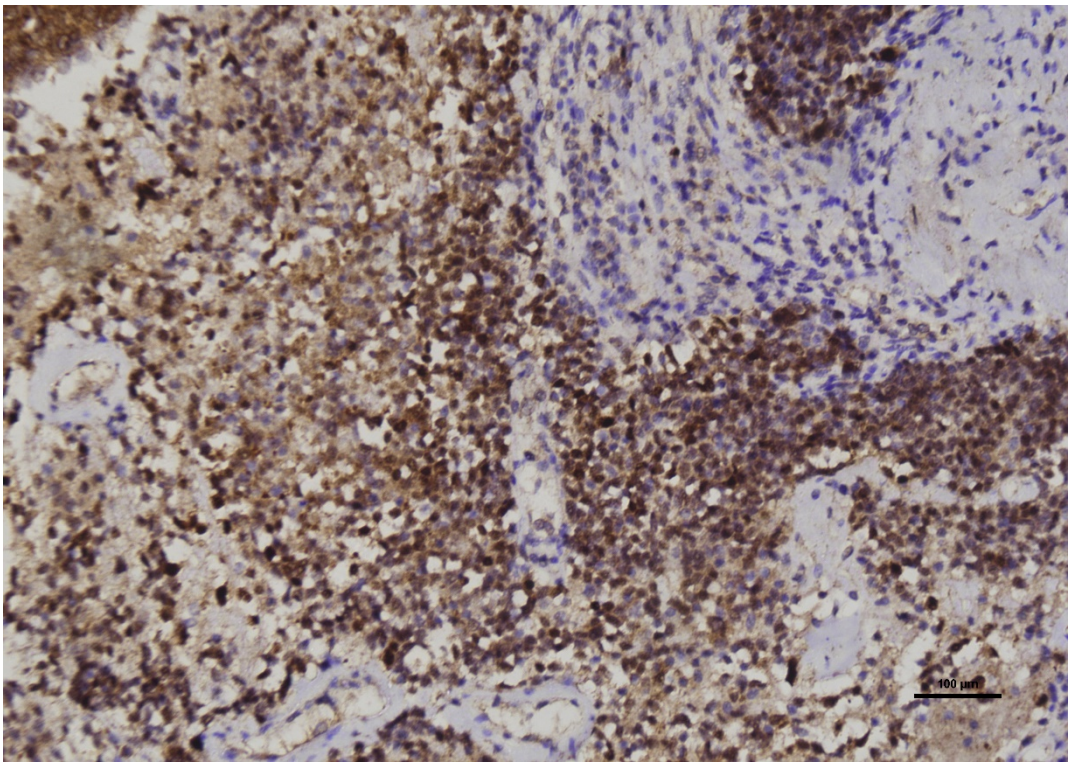


Figure 4.15 Invasive Cervical Carcinoma- of a 55-year-old female showing strong P16 Expression (IHC of same case as Figure 4.13, x20)

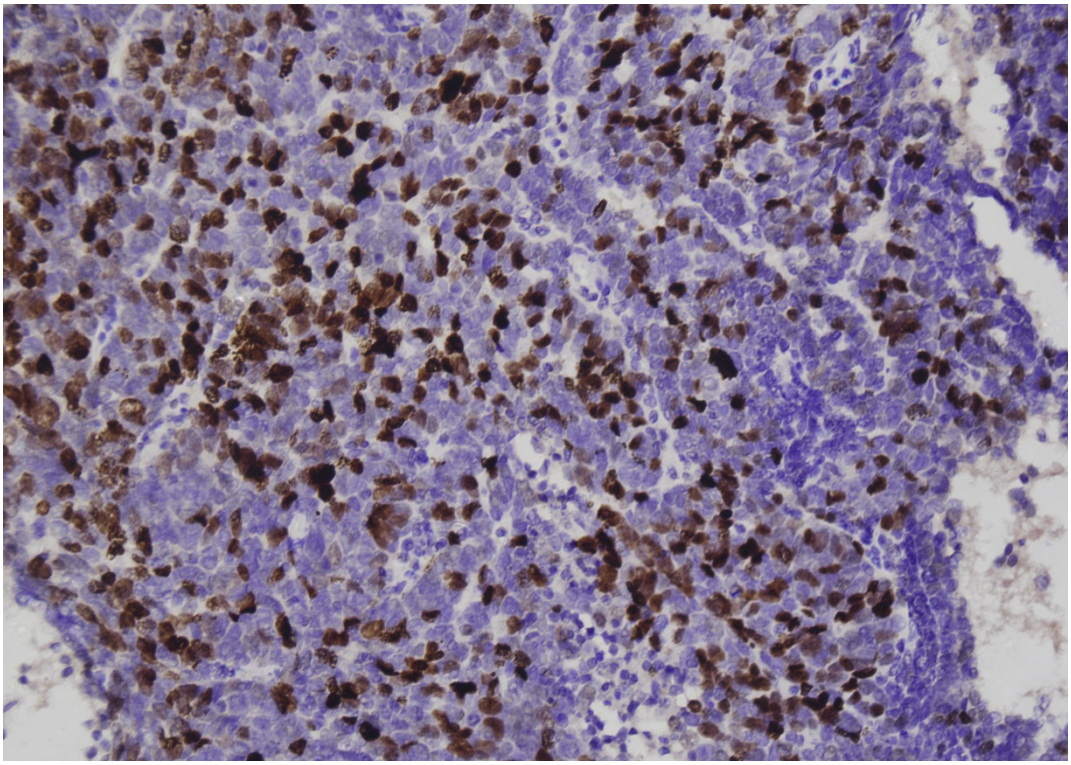


Figure 4.16 Invasive Cervical Carcinoma- of a 55-year-old female showing strong TOP2A Expression (IHC of same case as Figure 4.13, x20)

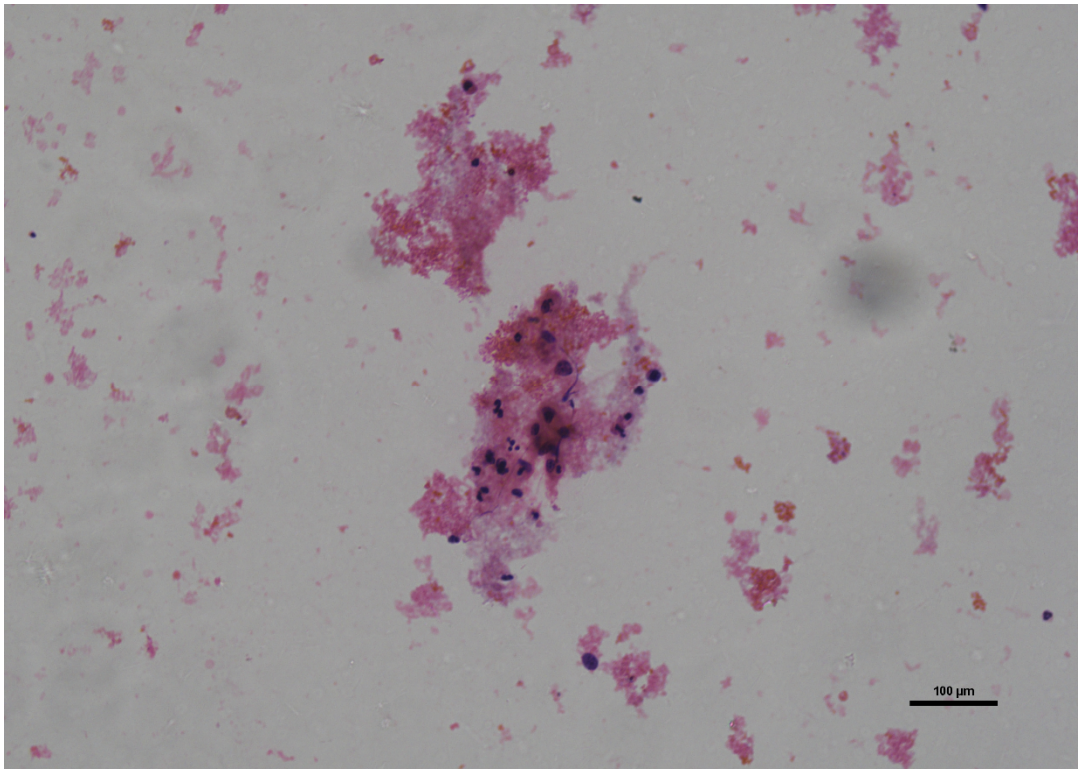


Figure 4.17 Atypical Glandular cells of a 60-year-old female (PAP, x 20)

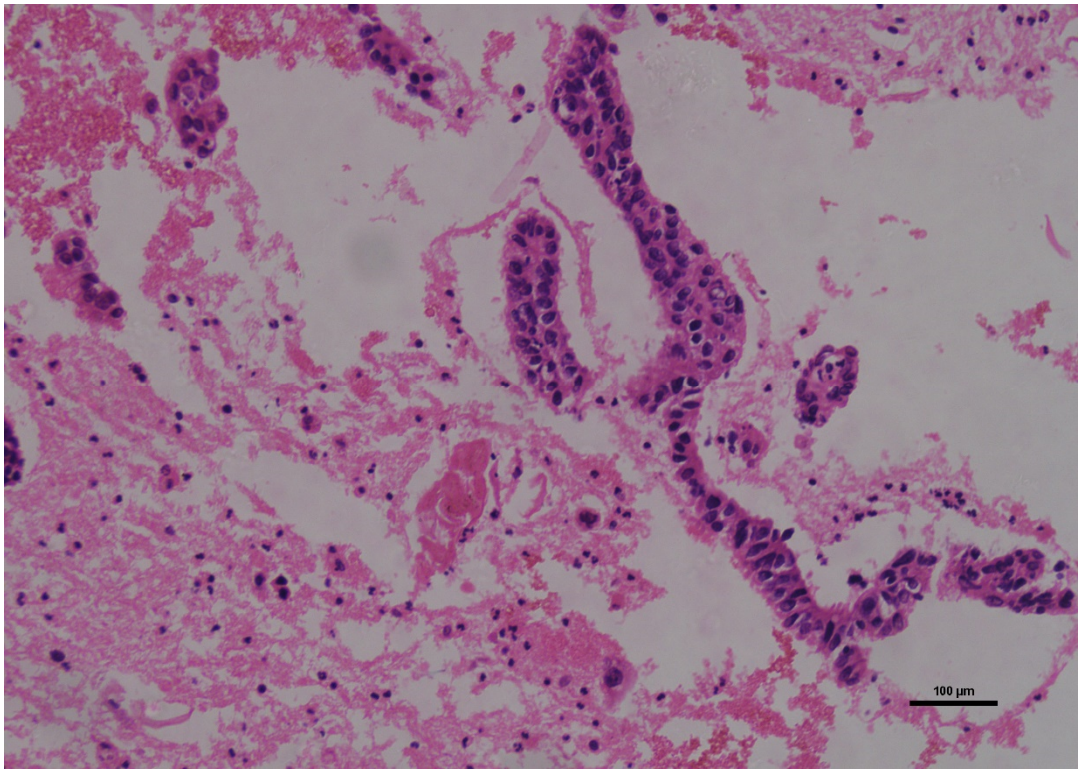


Figure 4.18 Atypical Glandular cells of a 60-year-old female (H&E of same case as Figure 4.17, x20)

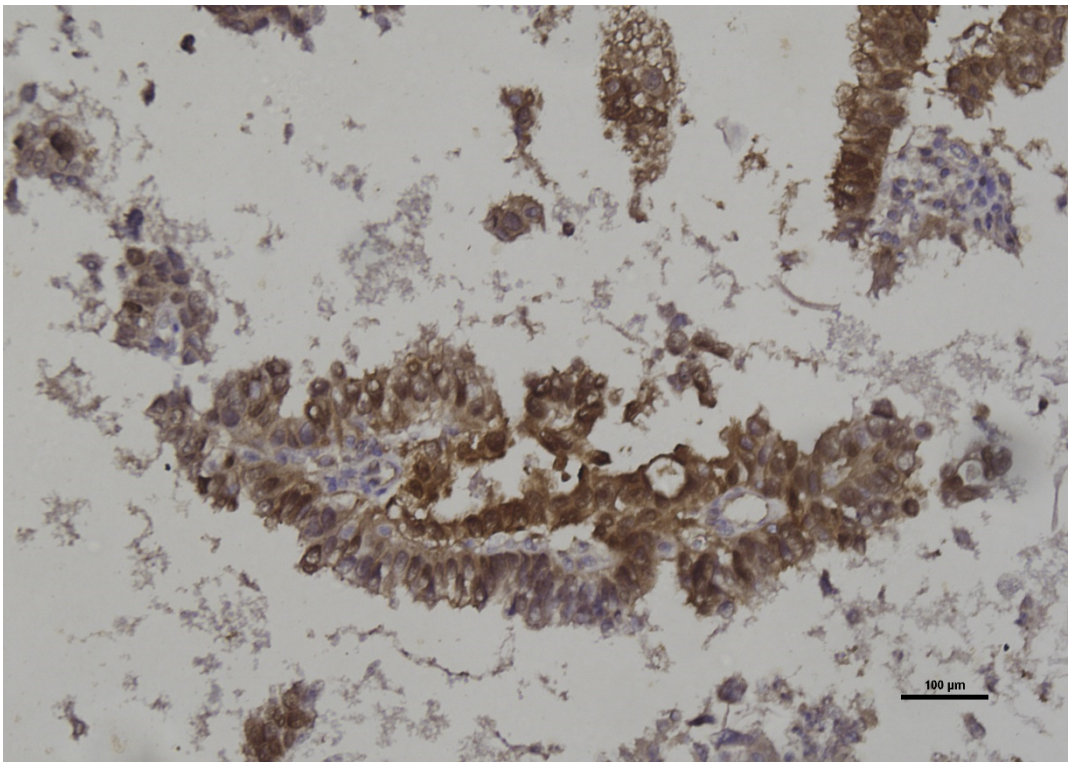


Figure 4.19 Atypical Glandular Cells- of a 60-year-old female showing moderate P16 Expression (IHC of same case as Figure 4.17, x20)

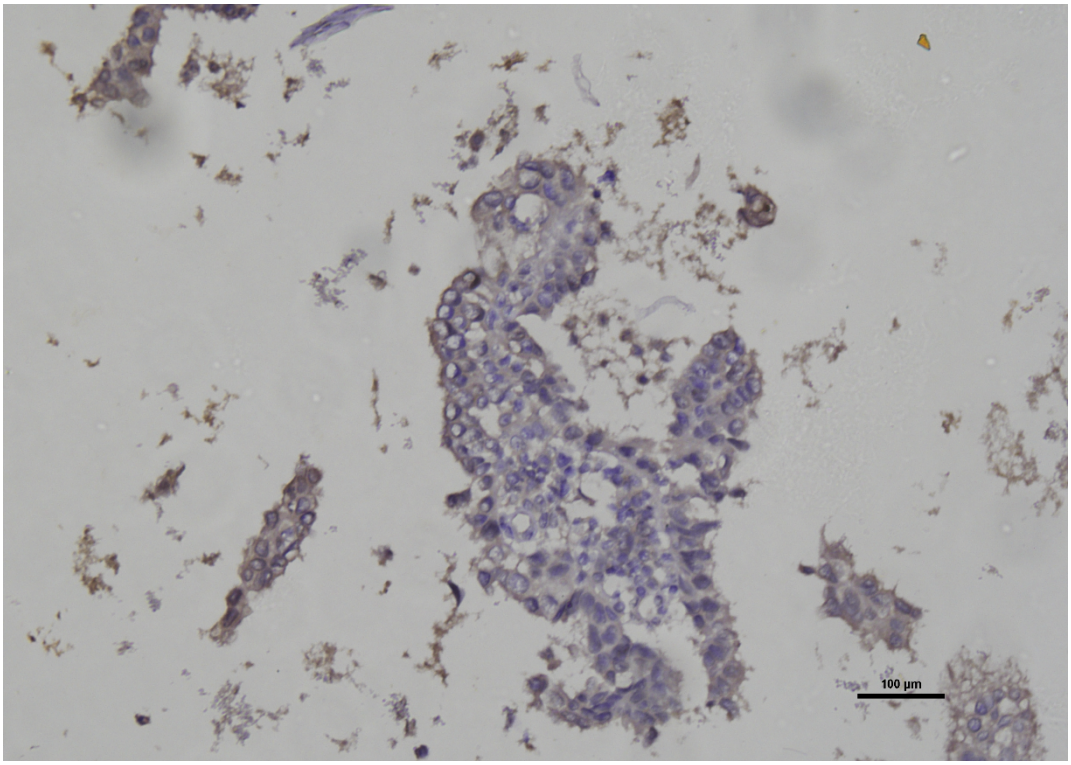


Figure 4.20 Atypical Glandular Cells- of a 60-year-old female showing no TOP2A Expression (IHC of same case as Figure 4.17, x20)

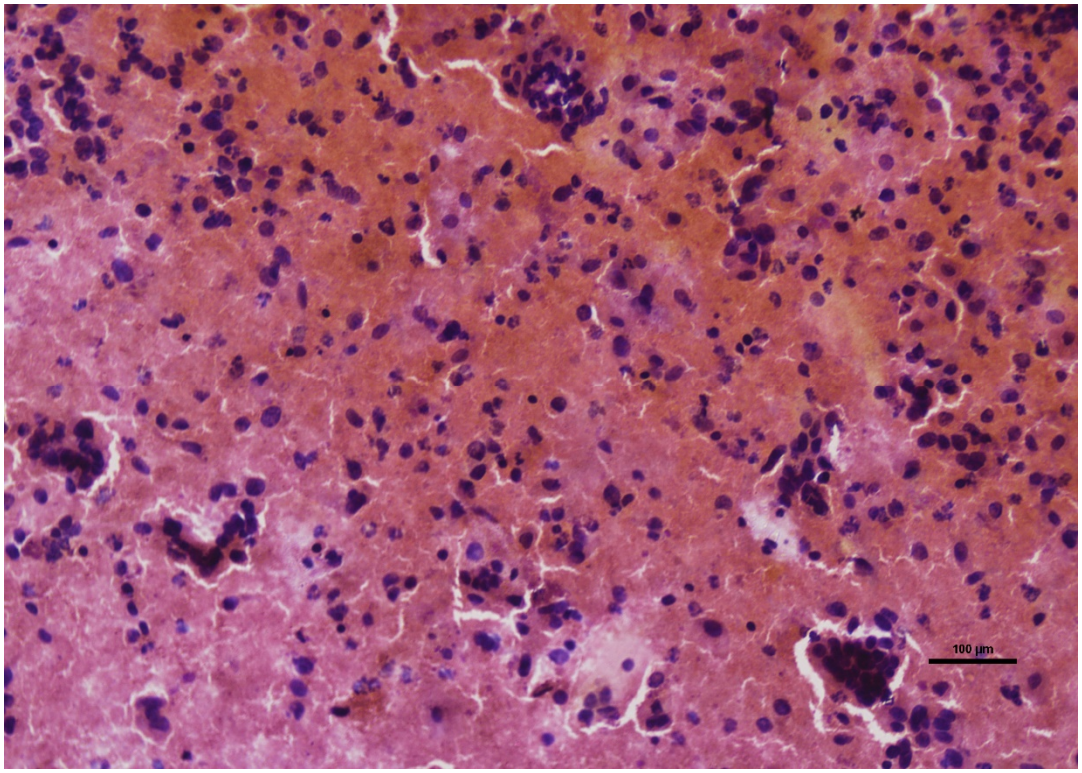


Figure 4.21 Invasive Cervical Carcinoma- of a 42-year-old female (PAP, x 20)

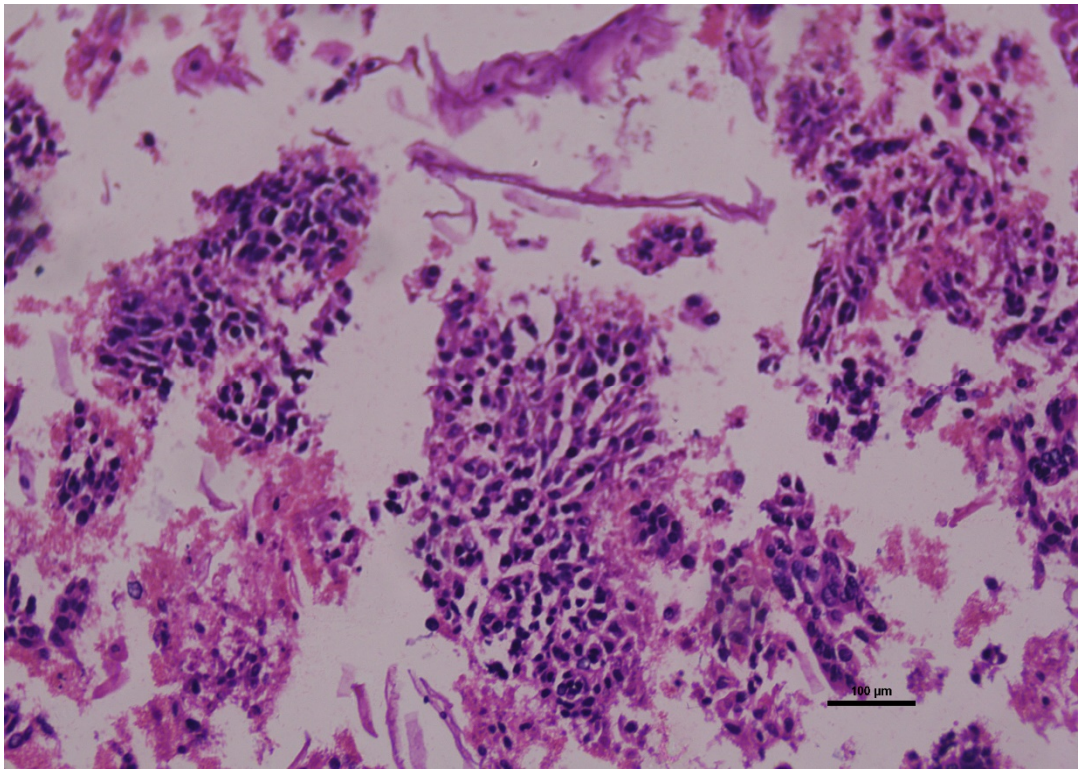


Figure 4.22 Invasive Cervical Carcinoma- of a 42-year-old female (H&E of same case as Figure 4.21, x20)

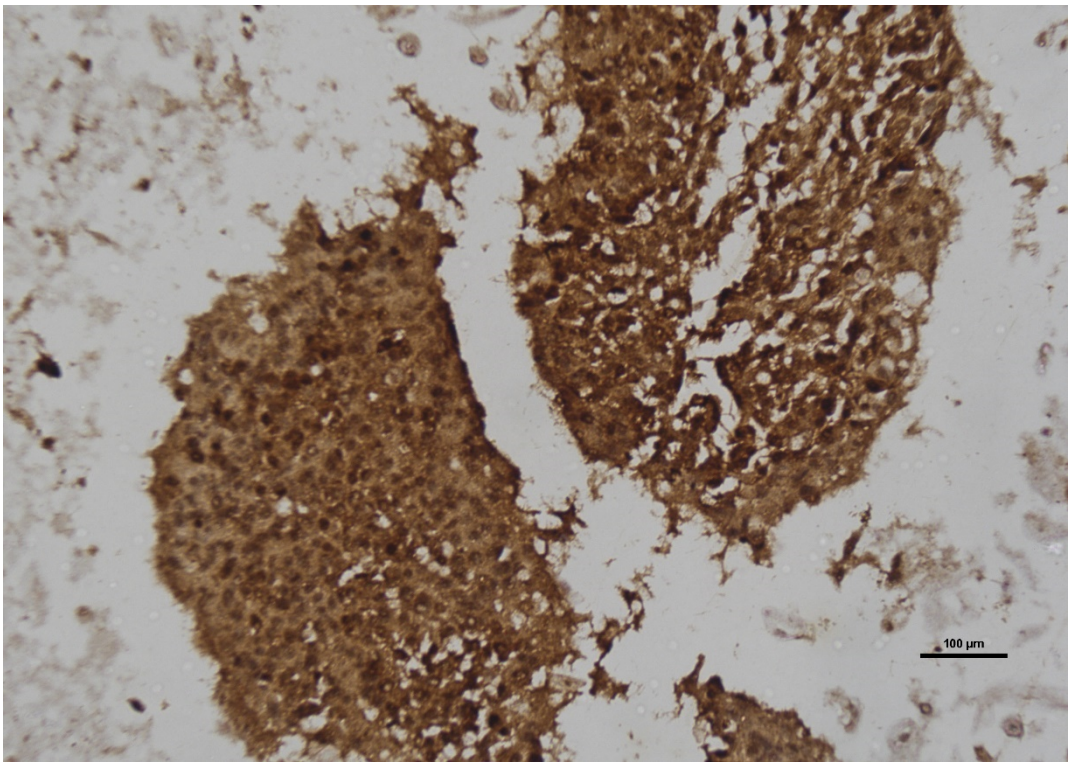


Figure 4.23 Invasive Cervical Carcinoma- of a 42-year-old female showing strong P16 Expression (IHC of same case as Figure 4.21, x20)

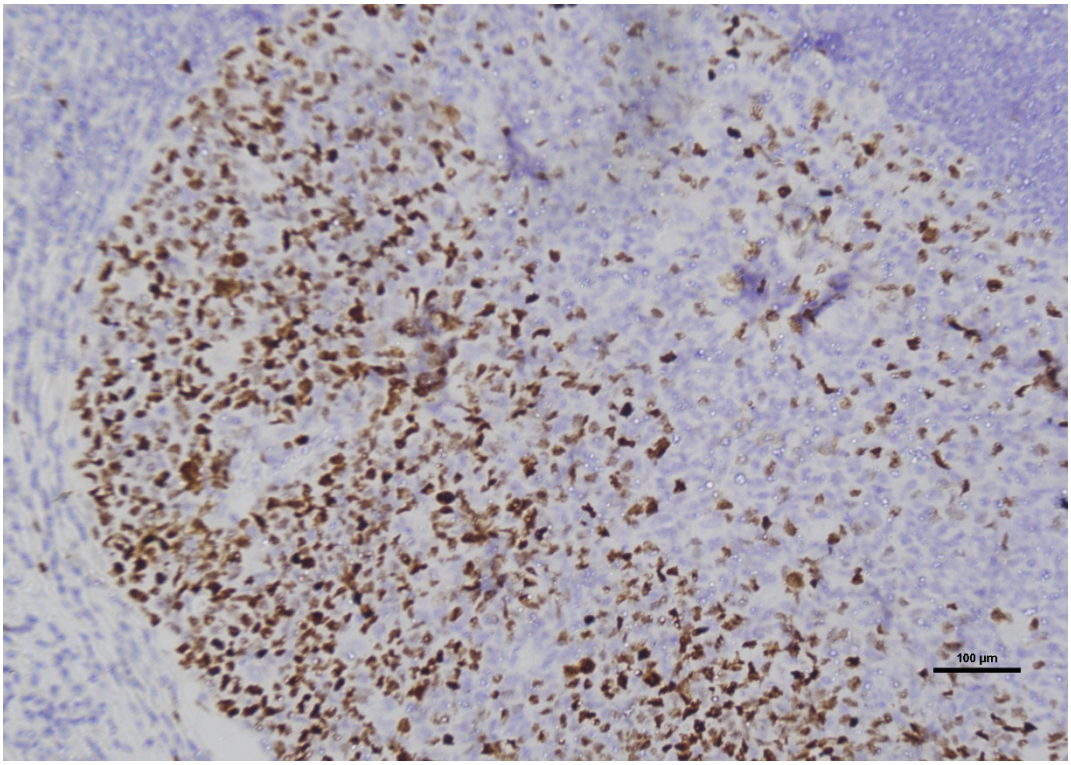


Figure 4.24 Invasive Cervical Carcinoma- of a 42-year-old female showing moderate TOP2A Expression (IHC of same case as Figure 4.21, x20)

CHAPTER 5

DISCUSSION & CONCLUSION

Cervical cancer is among the most common global gynaecological malignancy concern which has remained an imperative area of research in the last three decades. Better screening strategies, early detection, and standard of care can optimise outcomes for majority of cervical cancer patients. The main focus of this research was to analyse the level of P16 and TOP2A immunohistochemical expressions in various cervical precancerous and cancerous lesions and their correlation with clinicopathological parameters. As already mentioned, the rationale of this study was to determine the efficacy of these immunohistochemical markers in early diagnosis in the local population of Karachi, as well as to discover their correlation with various parameters. In this chapter, first the main findings and their implications will be explored, along with a comparison with similar studies done previously. Then the limitations and strengths of the study will be presented. Finally some future recommendations and concluding remarks will be made.

5.1 Sequence of Discussion Experiment Wise

5.1.1 Analysis based on Demographics

5.1.1.1 Age

In the current study, conducted from November 2023 to June 2024, a total of 60 Liquid Based Cytology samples were collected. The mean age of the patients was 46.23 years having a standard deviation of ±11.70 years. Majority of the patients, about 55%, were ≤45 years of age whereas 65% of the women were pre-menopausal. Notably, significant association of age categories as well as menopausal status is observed with cytological diagnosis and P16 expression levels, while TOP2A only demonstrates significant association with menopausal status. Furthermore, the mean age at Squamous

Cell Carcinoma (SCC) diagnosis was 56.8 years coinciding with the results presented by M. Wang et al., (2024) where the incidence of cervical SCC demonstrated an increasing curve with age, peaking at about 55 years. On the other hand, the mean age of adenocarcinoma diagnosis was 57.3 years, contradicting the same study M. Wang et al. (2024) in which the incidence of cervical adenocarcinoma displayed a gradual rise from 20–45 years, subsequently plateauing at >45 years of age group this discrepancy might be due to less number of adenocarcinoma cases in the studied population.

In terms of cytological diagnosis, 33 out of 60 patients were diagnosed as having non-cancerous lesion while 25 pre-cancerous and 2 cancerous lesions and the mean age of each group was 41.93, 51.72, 48.5 years respectively. These results show that women with >45 years of age group are more suspectable to cervical pre-cancerous and cancerous lesion consistent with the results of a previous study Shafique et al. (2024), where most pre-cancerous and CC lesions were prevalent among women of >40 years. The findings of the current research, therefore, substantiates the significance of regular CC screening among women of >45 years for detection and management of CC at an earlier stage.

5.1.1.2 Ethnicity

In the current sample space, the most common ethnicity group was Urdu speaking (31.7%) followed by Pakhtoon (23.3%), Sindhi (15%) and Punjabi (11.7%). All three biopsy proven adenocarcinoma cases were from Urdu speaking group while 4 out of 5 biopsy proven squamous cell carcinoma cases were from Pashto speaking group. There was a significant association detected between ethnicity and P16 immunohistochemical expression as shown in Table 4.10 highlighting the importance of targeted CC screening among high-risk population.

5.1.1.3 Education and Occupational Background

It was found that about 42% of patients received no formal education, whereas only 18.3% had completed elementary school, 13.3% had completed matric, and 8.3% had completed intermediate education. Merely 18% patients had graduation or higher degrees. Out of 60 women, 48 were housewives whereas only 12 had been working in different professions. Prior studies Kashyap et al. (2019); Thakur et al. (2015); Tebeu et

al. (2008); Shields et al. (2004) showed that less or no formal education, low socioeconomic status, being housewives, and women who had delivered first child at <20 years were associated with cervical lesions and cancer; however, no such association was found significant in our study.

5.1.1.4 Marital Status

More than 85% of the women who participated in this research were married. About 60% were married for more than 20 years and the majority of females (71.7%) were married at 15-20 years of age, coinciding with Idrak et al. (2024) where the mean age at marriage or sexual activity initiation was reported as 17.33 ± 4.73 years. Various other studies have revealed that marriage at a relatively younger age is a potential risk factor for cervical lesions Kashyap et al. (2019); Ruiz et al. (2012); Ogunbowale & Lawoyin (2008), however no relationship was found between marriage duration and age at marriage with cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression in our study.

5.1.2 Analysis based on Risk Factors

5.1.2.1 Age at Menarche and Menstrual Hygiene

In the current study, the average age at menarche was found to be 12.91±1.12 years. Out of 60 cases, 38(63.3%) females had average menstrual cycle lasting between 4-7 days, 56.7% of the women utilized reusable clothes while 43.3% used disposable pads. For majority of patients (more than 65%), the average number of menstrual hygiene products used in a day was less than 3, while for the rest, it was more than 3 per day. Although this clearly shows poor menstrual hygiene among a significant proportion of patients, the study did not find any significant association between the age at menarche and menstrual hygiene with the cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression. However, it is worth mentioning that previous studies Kashyap et al. (2019) and Thakur et al. (2015) have not only reported similar results but also concluded that early menarche i.e., age <13 years and poor menstrual hygiene are associated risk factors for cervical cancer.

5.1.2.2 Parity

In the current study, about two-thirds (63.3%) of the women had more than 3 children, with 7±4 children on average. No significant associations observed between high parity and cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression, which coincides with the results presented in H. Aziz et al. (2023) where no increased risk of HPV infection was linked with high parity. However, a number of other studies have shown contradictory results where high parity indeed has an association with CC and can be classified as one of the risk factors for developing cervical cancer in HPV positive patients (Abacjew-Chmyłk et al., 2016; Muñoz et al., 2002; Thakur et al., 2015). However, HPV status cannot be commented upon as no investigation for HPV identification was included in the study and no data regarding the same was available.

5.1.2.3 Smoking

The study has found only 8.3% (5) of the subjects having positive smoking history, while rest were non-smokers. The majority 4 out of 5 of women had prolonged smoking history for >5 years. Average Number of cigarettes smoked per day was 4.6±2.6 cigarettes. In our study no association was observed between history of smoking with biopsy diagnosis, P16 and TOP2A expression, which is consistent with Thakur et al. (2015) where no significant association was observed between smoking and CC. However, a significant association between cytological diagnosis and smoking status is observed as shown in Table 4.22, which substantiates a number of previous studies demonstrating a positive relationship between active tobacco smoking and cervical lesion development and malignant transformation (Jensen et al., 2012; Min et al., 2018; Shin et al., 2019; Su et al., 2018).

5.1.2.4 Multiple Sexual Partners

In our study, 10% women reported having multiple sexual partners (history of more than one marriage), whereas 18.3% had husbands with the history of multiple sexual partners (polygamous marriage). However, no significant association was found between history of multiple sexual partners or multiple sexual partners of husband with cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression. This can be due to patient

reluctance to give personal history regarding sexual behaviour of themselves and their spouse. A previous study by Thakur et al. (2015) has also reported no link between CC and having multiple sexual partners or multiple sexual partners of spouse. Nevertheless, a recent study has demonstrated that cervical cytological abnormalities i.e. ASC-US, LSIL, HSIL are more common among individuals who had history of multiple sexual partners (Umakanthan et al., 2023).

5.1.2.5 Urogenital Tract Infection

In our study, previous history of urogenital tract infection was discovered in 30 cases, where 60% women with positive history had cervicitis, 23.3% had recurrent UTIs, and 16.7% had both. Furthermore, no previous or current history of HIV or other STIs can be determined among these 60 patients. There was no association observed between previous urogenital tract infection history with cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression in our study.

5.1.2.6 Contraception Method

Out of total 60 patients, 23 had a history of using contraceptives, 13% used the barrier contraceptive technique, 26.1% used injectables, 34.8 % used IUDs, and 26.1 % used OCPS. Of these, 23 (38.3%) had a prolonged use history of oral contraceptives or other forms; 8.43±7.92 years was the average time spent using contraceptives. In our study no association was found between usage of OCPs or other forms with cytological diagnosis, biopsy diagnosis, P16 and TOP2A expression. This result is consistent with other previous studies by Thakur et al. (2015), and Kjellberg et al. (2000) where OCPs and other forms of contraception have not been reported as an independent risk factor for cervical lesions and cancer.

5.1.2.7 Prior Screening and Vaccination

During sample collection, it was noted that only 7 women had undergone Pap smear screening previously. Earlier studies have indicated a very low pap smear screening rate among developing countries, that is about 6.2% reported by Basu et al. (2014), consistent with the CC screening frequency in our study. Another study by Hirani et al.

(2021) conducted in Karachi reported 73% pap smear screening rate contradictory with our results as majority of the women in our study belong to low socio-economic background as compared to target population in that study. Furthermore, none of the women in the current study had been vaccinated against HPV due to limited awareness regarding CC and its preventable measures, while the same study reported that 9.8% of the women were vaccinated against HPV (Hirani et al., 2021).

5.1.3 Analysis based on Gynaecological Symptoms

In the current study, the most frequent gynaecological symptoms among the patients were lower abdomen pain (80%), postmenopausal bleeding (71.4%), abnormal vaginal discharge (56.7%) followed by dyspareunia (38.3%), intermenstrual bleeding (25%), and postcoital bleeding (20%). Similar results have been documented in the study by Aziz & Yousfani (2013), in which irregular vaginal bleeding, vaginal discharge, postmenopausal bleeding followed by pain in lower abdomen and post coital bleeding were the most common clinical symptoms reported in CC patients. Although no association was observed between any of these symptoms with biopsy diagnosis, P16 and TOP2A expression in our study, only dyspareunia was significantly associated with cytological diagnosis as shown in Table 4.23.

5.1.4 Analysis based on Biopsy Diagnosis

5.1.4.1 Determining Cancer Status

Out of the 60 samples collected in this study, only 12 had prior biopsy results available with 9 confirmed cancerous lesions. However, 7 of these cases belonged to post-menopausal women, among which 4 women had a positive history of post-menopausal bleeding. Overall, 5 biopsy confirmed cervical cancer cases were found to be Squamous Cell Carcinoma followed by Adenocarcinoma (3 cases) and only 1 poorly differentiated carcinoma. However, this contradicts the results presented in Loya et al. (2016) and Aziz & Yousfani (2013), where >90% of the cases were histologically classified as SCC. In global CC cases, Squamous Cell Carcinoma (SCC) is the predominant histologic subtype representing more than 80% cases while Cervical Adenocarcinoma contributing to only about 12% (M. Wang et al., 2024).

Furthermore, the cytological analysis of these cases align with the biopsy results. On cytology, the 9 biopsy confirmed cancerous lesions were diagnosed as ASC-US (1 case), HSIL (3 cases), Atypical Glandular Cells (3 cases) and Invasive Carcinoma (2 cases) respectively. Moreover, 7 of these cases displayed positive P16 expression while only 2 showed positive TOP2A expression.

5.1.4.2 Determining Biomarker Expression Levels

The 5 biopsy proven SSC cases displayed varied P16 expression levels. Specifically one sample each showed no, mild and moderate expressions whereas only 2 samples displayed high expressions. On the other hand, the 3 adenocarcinoma cases equally distributed as having mild, moderate and high P16 expression levels. Nevertheless, these results appear to contradict a similar study by Zuberi et al. (2021) in which 100% of SCC showed high P16 expression while more than 80% adenocarcinoma cases displayed high P16 expression.

With regards to TOP2A expression levels, only 2 out of 5 biopsy proven SCC cases displayed moderately positive expression whereas all 3 adenocarcinoma cases were found to have no expression. Once again, these results contradict Zuberi et al. (2021) where 60.2% of SCC showed high TOP2A expression, 27.7% showed moderate TOP2A expression and 12% showed mild TOP2A expression. While in the case of adenocarcinoma, the same study by Zuberi et al. (2021) suggests mild TOP2A expression in about 16.7% cases, and high TOP2A expression levels in rest of the cases. This discrepancy is potentially due to relatively less number of cancerous cases (9 cases) included in the current study as compared to more than 90 cancerous case in the previous study.

For completeness, the only poorly differentiated carcinoma case did not show P16 and TOP2A expressions. Furthermore, the 3 biopsy confirmed benign lesions did not express TOP2A but indeed displayed mild to moderate P16 expression levels.

5.1.4.3 Determining FIGO Staging

In the present study, FIGO clinical staging of 9 biopsy proven cancer cases was done and patients were classified as having stage I-B, stage II-A, stage II-B (2 cases each), and stage III-A (3 cases) respectively. Nevertheless due to less number of available cases, no clear association was found between FIGO staging and the P16, TOP2A expression levels consistent with an older study by Davidson et al. (2000) in which TOP2A was neither associated with staging nor grading in cervical cancer cases. In contrast, prior studies have indeed demonstrated that positive P16 expression has better tumor differentiation and perhaps a better prognostic indication in cervical cancer patients (J. Lin et al., 2014; J. Wu et al., 2024).

5.1.5 Analysis based on Cytological Diagnosis

5.1.5.1 Determining Cancer Status

Out of the 60 samples analyzed cytologically, 33 were found to be non-cancerous, whereas 25 had pre-cancerous lesions, and only 2 had cancerous lesions. Furthermore, the mean ages of these patient groups were 41.93±8.66 years among the non-cancerous, 51.72±13.27 years among the pre-cancerous, and 48.50±9.19 years among the cancerous patients. Only 2 out of the 33 non-cancerous samples displayed mild to moderate P16 expression, whereas only 6 cases from the 27 pre-cancerous/cancerous group exhibited dual staining for P16 and TOP2A, while 14 cases displayed P16 expression only. The remaining 7 cases from the pre-cancerous group that did not show P16 expression level were classified as 50% (5 cases) ASC-US, 20% (1 case) LSIL and 16.7% (1 case) HSIL. Furthermore, the 21 samples from the pre-cancerous group that also did not show TOP2A expression were categorized as 90% (9 cases) ASC-US, 60% (3 cases) LSIL, 83.3% (5 cases) HSIL and 100% (4 cases) Atypical Glandular Cells.

As a result, a significant association was observed between cancer status and P16, TOP2A expressions in our study although it is important to highlight that number of cancerous lesions had negligible weightage as compared to non-cancerous and pre-cancerous lesion. However, this contradicts a previous study by Zuberi et al. (2021) in which no significant association was found, though it is worth emphasizing that both

biomarkers were applied to FFPE biopsy tissue which was not the case in the current study. Therefore based on the current analysis, it is concluded that both P16 and TOP2A immunohistochemical markers can potentially be utilized as an effective diagnostic tool across all age groups to validate cytological diagnosis.

5.1.5.2 Determining Cytological Abnormalities

The cytological diagnosis of the 60 examined samples is as follows:

- The non-cancerous cases were classified as 26.7% (16 cases) NILM, and 28.3% (17 cases) Inflammatory.

- The pre-cancerous cases were categorized as 16.7% (10 cases) ASC-US, 6.7% (4 cases) Atypical Glandular Cells, 8.3% (5 cases) LSIL, and 10% (6 cases) HSIL.

- The 2 cancerous cases (3.3% of total cases) were identified as Invasive Carcinoma.

Note that the P16 and TOP2A immunostaining was conducted on cytology cell blocks which demonstrated increasing expression level with the increasing severity of the cytological abnormality. Evidently, there exists a significant association between the cytological diagnosis and both P16, TOP2A expression levels.

Overall, it was found that 36.7% patients had positive P16 expression, while only 10% had positive TOP2A expression. This is unlike a previous study by Zuberi et al. (2021) which had reported significantly higher positivity rates (about 70% for P16 and 60% for TOP2A) among normal, pre-cancerous and cancerous biopsies. This contradiction has been observed possibly due to a relatively larger sample size as well as employing a relaxed positive expression criteria for both immunohistochemical markers applied on cervical tissue. More specifically, that study considered P16 positive staining either in the nucleus or cytoplasm leading to higher positivity rate as compared to the current research, in which positive P16 staining was considered only when there was only

nuclear staining or both the nucleus and cytoplasm stained positive together (Liu et al., 2017; Shelton et al., 2017).

Cytological diagnosis was conducted using Bethesda classification, the detailed results for P16 expressions are as follows:

- Total 16 cases were diagnosed as NILM, in which only 1 case (6.25%) showed moderate P16 expression level.

- 17 cases were diagnosed as inflammatory, with only 1 (5.8%) displaying mild P16 expression.

- 10 cases were diagnosed as ASC-US, only 50% of these cases displayed positive P16 immunohistochemical expression, 4 of which showed moderate P16 expression while the remaining 1 showed mild P16 expression.

- Total 5 cases were diagnosed as LSIL, out of which 4 (80%) had positive P16 expression (3 exhibited mild while 1 exhibited moderate P16 expression) and 1 case exhibited no P16 expression.

- 6 cases were diagnosed as HSIL, with 5 (83.3%) demonstrating positive P16 expression (2 mild and 3 moderate), while 1 case showed no P16 expression.

- 4 cases were diagnosed as Atypical Glandular Cells, out of which 1 demonstrated mild, 2 demonstrated moderate, and 1 demonstrated high P16 expression, i.e. 100% positivity rate.

- Only 2 cases were diagnosed as invasive carcinoma and both of them illustrated high P16 expression, i.e. 100% positivity.

More or less similar results were suggested in the systemic review and meta-analysis conducted by Tsoumpou et al. (2009) where P16 positivity rate among normal,

ASC-US, LSIL, and HSIL cytology smear were 12%, 45%, 45% and 89% respectively. Whereas on histology, 2% normal tissue, 38% CIN1 lesion, 68% CIN2 lesions and 82% CIN3 high grade lesions had positive P16 immunohistochemical expression. In another study Liao et al. (2014) reported that, the positivity rate of P16 expression in 6557 cytologically and histologically confirmed cases with normal, CIN1, CIN2 CIN3 and cancerous lesion were 2.7 %, 42.7 %, 75.5 %, 79.6 % and 100 % respectively. One more study by Kanthiya et al. (2016) has demonstrated increasing P16 expression level with the increasing lesion severity; positivity rates were 9.4% in non-dysplastic, 10.4% in CIN1 lesion, 78.7% in CIN2/3 lesion while 91.3% in invasive carcinomas. Another study by Ghosh et al. (2020) reported absolute P16 positivity in 26.3% cases of LSIL while 58.3% cases of HSIL. A similar trend was revealed in a recent study by Rezhake et al. (2021) in which the positivity rate of P16 increases as severity of lesion increases 7.0% for benign, about 50% for CIN1, 84.5% for CIN2, and 90.3% for CIN3 and invasive cancer. However, in the latest study by Hosseini et al. (2023) P16 was negative in all of the LSIL cases while, 58.7% HSIL diagnosed case had positive P16 expression. Although the available literature, as well as the current study, suggests a strong P16 immunostaining association with the severity of cervical cytological or histological abnormalities, the global reproducibility of these results is limited possibly due to the lack of standardized immunostaining interpretation between pathologists. In order to improve P16 immunostaining's and reduce interpretation bias across the globe, standardized protocols and the use of high-quality, validated antibodies are crucial. Besides, strict quality control techniques, i.e. the use of positive and negative control samples are required. Lastly, encouraging data exchange and transparency among researchers, can greatly enhance results' reliability and consistency.

In the case of TOP2A, positive expression was only noticed in cervical pre-cancerous and cancerous lesions, consistent with a previous study Davidson et al. (2000) in which no immunoreactivity for TOP2A was observed among control group. More specifically, the following results were obtained in our study:

- Only 1 out of 10 ASC-US cases displayed mild TOP2A immunohistochemical expression.

- Similarly, 2 out of 5 LSIL cases exhibited mild TOP2A expression.

- Only 1 out of 6 HSIL cases demonstrated mild TOP2A expression.

- Among the 4 diagnosed Atypical Glandular Cells cases, none demonstrated TOP2A expression.

- Both cases of Invasive Carcinoma exhibited moderate TOP2A expression.

In comparison, a study by Peres et al. (2016) has reported positive TOP2A expression in 35.9% of NILM, 40% of ASC-US, 68.4% LSIL, and 100% of HSIL contradicting our results. Another study by Zuberi et al. (2021) evaluated TOP2A expression in pre-cancerous and cancerous tissue; 6 out of 7 CIN1 lesions showed mild TOP2A expression while only 1 showed moderate TOP2A expression, 1 CIN2 was diagnosed which displayed high TOP2A expression, 2 out of 3 CIN3 lesions showed mild TOP2A expression while the remaining 1 showed moderate TOP2A expression. Although it is important to mention that TOP2A was applied to FFPE biopsy tissue which was not the case in the current study.

Out of the 27 pre-cancerous and cancerous cervical cases identified in our study, only 6 cases demonstrated positive staining for both P16 and TOP2A antibodies indicating HPV infection. Specifically, 1/10 ASC-US, 2/5 LSIL, 1/6 HSIL, and 2/2 Invasive Carcinoma exhibited dual staining for P16 and TOP2A. This result is considerably smaller than the one suggested in a previous study by Shi et al. (2007) which explored the dual immunostaining of P16 and ProExC on cervical biopsy tissue, concluding that 100% of both LSIL and HSIL cases demonstrate dual positivity. This implies that the combining P16 with TOP2A (a component of ProExC) has potentially better diagnostic properties in detecting the pre-cancerous lesions, i.e. LSIL and HSIL if applied on FFPE biopsy tissue.

5.1.6 P16 and TOP2A Diagnostic Performance

The diagnostic performance of both P16 and TOP2A was evaluated by computing sensitivity, specificity, predictive values, and diagnostic accuracy using the cervical lesion as the standard.

5.1.6.1 P16 Sensitivity, Specificity, Predictive Values, and Diagnostic Accuracy

With regards to P16 expression, 20 patients were true positive who were correctly diagnosed as positive having pre-cancerous or cancerous lesion, while 31 non-cancerous patients were correctly diagnosed as negative. The sensitivity, specificity, PPV, NPV and accuracy of P16 in detecting pre-cancerous and cancerous lesions were 74.1%, 93.9%, 90.9%, 81.6% and 85% respectively. In terms of the P-values, 0.000 for sensitivity, specificity, PPV, NPV and accuracy all of which were < 0.05 (showing statistical significance).

These results coincide with the previous study conducted by Kanthiya et al. (2016) in which the sensitivity and specificity of P16 for detecting >CIN2 lesion were 84.5% and 90.5%. Another study by Rezhake et al. (2021) reported that the sensitivity and specificity of P16 were 88% and 92.3% respectively which are relatively consistent with the current study. However, a recent study by Ghosh et al. (2020) showed contradictory findings in which the sensitivity, specificity, positive predictive values, and negative predictive values of P16 expression for squamous intraepithelial lesions were 38.7%, 100%, 100% and 51.3% correspondingly. Similarly, Hosseini et al. (2023) reported 58.73% sensitivity, 100% specificity, 100% positive predictive values, 72.79% negative predictive values of P16 among pre-cancerous lesions i.e. LSIL and HSIL. Another study by Zuberi et al. (2021) reported P16 expression sensitivity: 97.2%, specificity: 91.3%, accuracy: 92.8% for cervical pre-cancerous and cancerous biopsy. Similarly, F. Zhao et al. (2022) evaluated P16 expression on LBC samples and reported the sensitivity, specificity, positive predictive values, and negative predictive values as 96.90%, 88.72%, 58.96% and 99.42% respectively, which is fairly consistent with the results of the current study. Besides M.-Z. Wu et al. (2019) had reported that the diagnostic performance of P16 immunostaining was way better than cytology with sensitivity of 96.0%, specificity 88.2% and accuracy of 91.7% respectively.

5.1.6.2 TOP2A Sensitivity, Specificity, Predictive Values, and Diagnostic Accuracy

In case of TOP2A expression, 6 patients were true positive, correctly diagnosed as positive having pre-cancerous or cancerous lesion and 33 patients were true negative, correctly diagnosed as negative. Sensitivity, Specificity, PPV, NPV and accuracy of TOP2A were 22.2%, 100%, 100%, 61.1% and 65% respectively. Similar study by Zuberi et al. (2021) reported TOP2A expression sensitivity: 77.6%, specificity: 93.3%, and accuracy: 87.8% for cervical pre-cancerous and cancerous biopsy. Another study by Tosuner et al. (2017) evaluated ProEx C (TOP2A/MCM2 combo) immunostaining on liquid based cytology samples and reported that the overall sensitivity of the marker in detecting squamous intraepithelial lesion was 40% whereas specificity was 100% consistent with the current study. In contrast, a slightly older study Shroyer et al. (2006) demonstrated 100% sensitivity as well as specificity of ProEx C for HSIL high grade lesion, contradicting the current research results.

5.1.7 Summary

In summary, a significant role of P16 and TOP2A immunohistochemical expression for detecting cervical lesions has stood out in this study. A strong association of P16 and TOP2A immunohistochemical expression with menopausal status, cytological diagnosis, and cancer status has been identified. Strong P16 immunostaining was directly associated with the increasing severity of cervical cytological abnormalities. Whereas TOP2A immunostaining is specific and might be a better tool for ruling out non-cancerous lesion.

Although the utility of these immunohistochemical markers, i.e. P16 and TOP2A, may somewhat increase the screening tests cost in relation to HPV genotyping as triage but it can lower the unnecessary colposcopy and biopsy referral rate as well as decrease the repeated cytology follow up burden. Hence, it could be an efficient as well as cost-effective method for detecting early pre-cancerous lesion and distinguishing early lesion from advance cancerous lesions in the long run. Furthermore, the high-throughput and easy-to-interpret results provided by these immune markers can probably serve as an important screening tool for larger screening programs.

5.2 Implications of the Study

5.2.1 Theoretical Implications

- P16 is a reliable immunohistochemical marker for detecting cervical pre-cancerous and cancerous lesion if applied with a stringent positivity criteria

- Rate of P16 expression positivity was directly associated with the degree of cervical dysplasia

- TOP2A expression level was observed in the advanced lesions which might be negative for another proliferative marker i.e. Ki-67

- Both the marker had positive association with menopausal status which demonstrates better screening potential in high-risk groups

5.2.2 Practical Implications

- P16 can be used as diagnostic panel marker to identify cervical precancerous and cancerous lesion at an earlier stage while TOP2A can be used as a diagnostic marker for advanced cancerous cases along with the cytology

- HPV testing along with cytological and immunohistochemical analysis should be considered necessary for cervical cancer risk identification in our population

- Arrangements could be made to replace conventional Pap smear with LBC which is a more efficient method of CC screening

5.2.3 Policy Implications

- Public health awareness seminars and campaigns should be conducted to educate women regarding cervical cancer symptoms, risk factors, preventive measures

and importance of regular screening for early detection and management of the disease

- Organization of extensive HPV vaccination campaigns covering various high-risk genotypes among teenagers to prevent HPV-related disease and cervical cancer

- Easy access and affordable cervical cancer screening services should be implemented incorporating both cytological and HPV testing for early detection of pre-cancerous lesions

5.3 Limitations and Strengths of Study

5.3.1 Limitations

- The sample size was small therefore generalization of the results cannot be done

- This cross-sectional study was conducted at only two tertiary care hospital and therefore will not be a true representative as the sample represents an institutional selection bias

- Long-term follow-up was not done on patients with strong expression levels of P16 and TOP2A so prognosis cannot be assessed directly

- HPV genotyping as well as molecular analysis on pre-cancerous and cancerous cases was not performed

- Tissue biopsy was not done for patients who were diagnosed as pre-cancerous on cytology, therefore their histological diagnosis cannot be determined

5.3.2 Strengths

- Lesions at various stages of neoplastic transformation i.e. ASC-US, Atypical Glandular Cells, LSIL, HSIL and cervical cancer was subjected to IHC, thus giving an insight into the differential expression of these biomarkers in relation to pre-cancerous and cancerous lesions

- The study included high-risk subjects belonging to different areas and ethnic group of Pakistan, and therefore provides valuable insights about the target population susceptible to developing cervical lesions

- Immunohistochemical expression criteria was very stringent, therefore no bias can be made

- Multiple parameters were taken into account so as to evaluate the association with cytological diagnosis and immunohistochemical expression

- Multiple demographics and known cervical cancer risk factors were evaluated with cytological diagnosis to know about prevalent risk factors in our study

5.4 Future Research Directions / Recommendations

- Based on the availability of cases more detailed study with larger sample size and molecular analysis would be needed to substantiate the results

- Large-scale clinical trials are needed to determine the sensitivity and specificity of these biomarkers in detecting pre-cancerous lesions and early-stage cervical cancer by Immunohistochemistry (IHC). Additionally, standardized protocols for detecting and measuring these biomarkers need to be established to ensure consistency and reproducibility across different laboratories

- A wide scale nationwide cervical cancer awareness program should be organized to decrease the prevalence of such preventable cancer

- Cervical cancer screening protocols and recommendations should be designed specifically among high-risk patients in our population

5.5 Conclusion

To our knowledge this study is the first to assess the association of various clinicopathological parameters with cytological diagnosis and differential expression of P16 and TOP2A among high-risk groups in Karachi, Pakistan.

- This study demonstrates significant association of P16 and TOP2A immunohistochemical expression with menopausal status, cytological diagnosis, and cancer status

- Strong P16 immunostaining was directly associated with the severity of cervical cytological abnormalities

- The sensitivity and specificity of P16 for detecting pre-cancerous and cancerous lesion was significantly higher, whereas TOP2A demonstrated higher specificity for detecting cancerous lesions

- P16 can be a reliable immunohistochemical marker for diagnosing early as well as late cancerous lesion while TOP2A is highly specific marker for ruling out non-cancerous lesion

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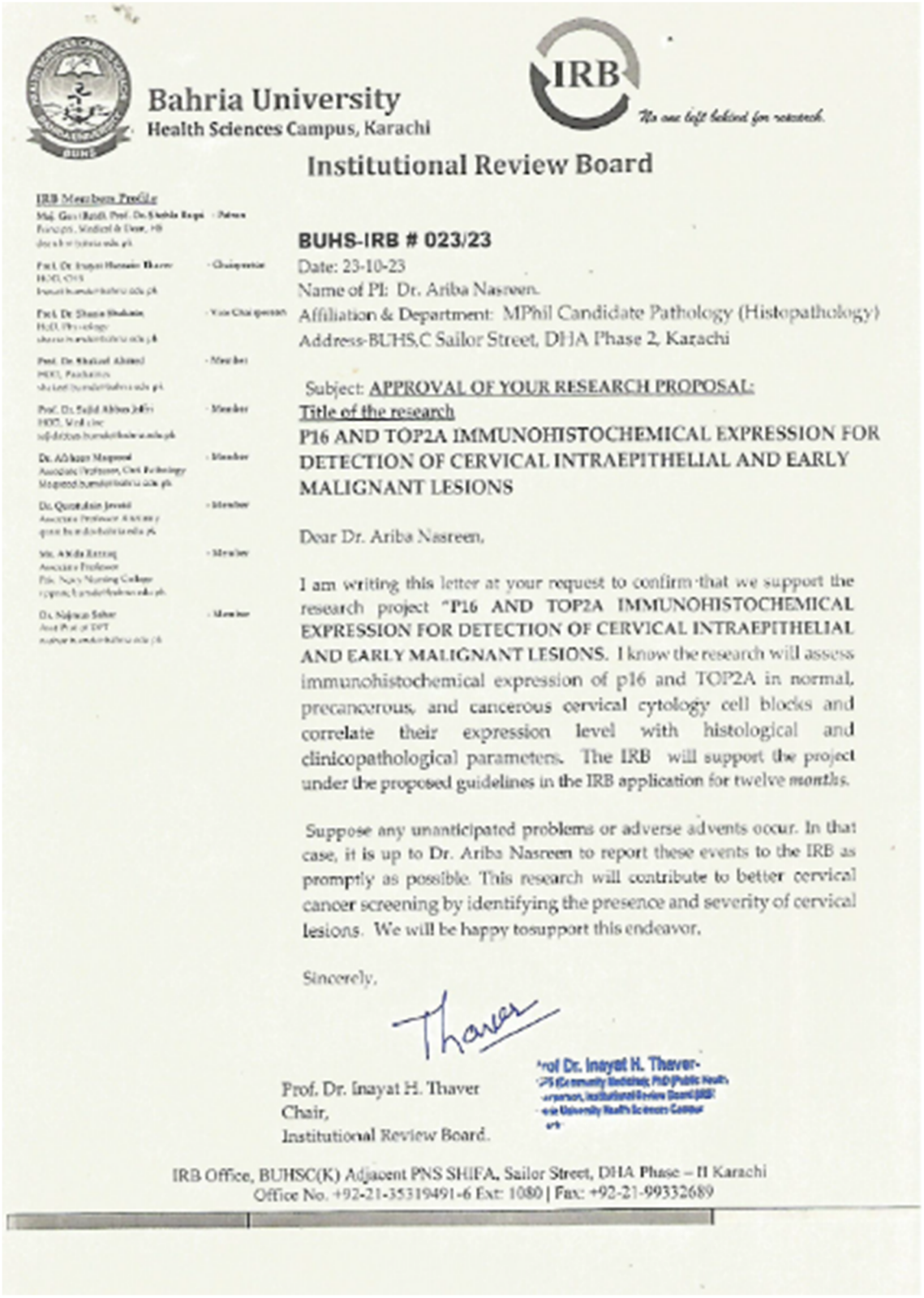
A. IRB- BAHRIA UNIVERSITY



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Health Sciences Campus, Karachi

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BUHS-IRB # 023/23

Date: 23-10-23

Name of PI: Dr. Ariba Nasreen.

Affiliation & Department: MPhil Candidate Pathology (Histopathology)

Address-BUHS,C Sailor Street, DHA Phase 2, Karachi

Subject: APPROVAL OF YOUR RESEARCH PROPOSAL:

Title of the research

P16 AND TOP2A IMMUNOHISTOCHEMICAL EXPRESSION FOR DETECTION OF CERVICAL INTRAEPITHELIAL AND EARLY MALIGNANT LESIONS

Dear Dr. Ariba Nasreen,

I am writing this letter at your request to confirm that we support the research project "**P16 AND TOP2A IMMUNOHISTOCHEMICAL EXPRESSION FOR DETECTION OF CERVICAL INTRAEPITHELIAL AND EARLY MALIGNANT LESIONS.** I know the research will assess immunohistochemical expression of p16 and TOP2A in normal, precancerous, and cancerous cervical cytology cell blocks and correlate their expression level with histological and clinicopathological parameters. The IRB will support the project under the proposed guidelines in the IRB application for twelve *months*.

Suppose any unanticipated problems or adverse advents occur. In that case, it is up to Dr. Ariba Nasreen to report these events to the IRB as promptly as possible. This research will contribute to better cervical cancer screening by identifying the presence and severity of cervical lesions. We will be happy tosupport this endeavor.

Sincerely,

Thaver

Prof. Dr. Inayat H. Thaver
Chair,
Institutional Review Board.

Prof. Dr. Inayat H. Thaver
Chairperson, Institutional Review Board (IRB)
Bahria University Health Sciences Campus
Karachi

IRB Office, BUHSC(K) Adjacent PNS SHIFA, Sailor Street, DHA Phase – II Karachi
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B. IRB- DOW UNIVERSITY

Dow University of Health Sciences
Institutional Review Board (IRB)

Ref: IRB-3172/DUHS/Approval/2023/ 418 Dated: 31st October, 2023

Prof. Dr. Naseem Ahmed Sheikh
Department of Pathology,
Dow Medical College,
Dow University of Health Sciences.

Subject: Institutional Review Board's approval for a research proposal.

Title of Study: P16 and Top2a Immunohistochemical Expression for Detection of Cervical Intraepithelial and Early Malignant Lesions

CRR Due Date: 30th October, 2024

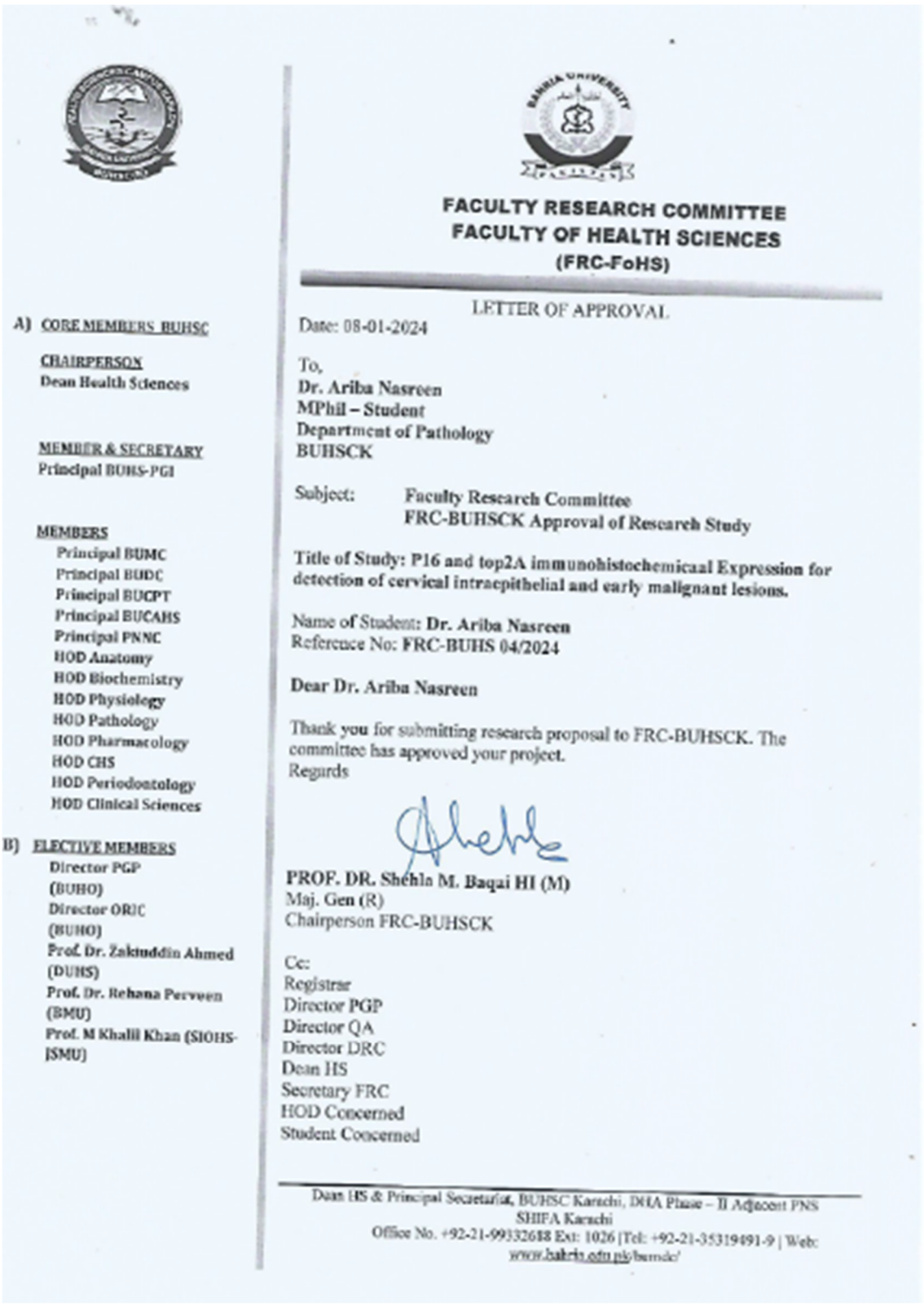
Dear Prof. Dr. Naseem Ahmed Sheikh,

Thank you for submitting the above mentioned study proposal. I am pleased to inform you that the IRB-DUHS has reviewed this proposal in its **197th meeting** held on **02nd September, 2023** and gives approval for a period of one year to conduct this study.

Any change in the protocol or extension in the period of study should be notified to the board for approval. Interim report on progress of study should be submitted to IRB from time to time.

Prof. Kashif Shafique
Professor of Public Health,
Chairman Institutional Review Board,
Dow University of Health Sciences,
Karachi.

C. FRC- BAHRIA UNIVERSITY



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LETTER OF APPROVAL

Date: 08-01-2024

To,
Dr. Ariba Nasreen
MPhil – Student
Department of Pathology
BUHSCK

Subject: **Faculty Research Committee**
FRC-BUHSCK Approval of Research Study

Title of Study: P16 and top2A immunohistochemicaal Expression for detection of cervical intraepithelial and early malignant lesions.

Name of Student: **Dr. Ariba Nasreen**
Reference No: **FRC-BUHS 04/2024**

Dear Dr. Ariba Nasreen

Thank you for submitting research proposal to FRC-BUHSCK. The committee has approved your project.
Regards

PROF. DR. Shehla M. Baqai HI (M)
Maj. Gen (R)
Chairperson FRC-BUHSCK

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D. WRITTEN INFORMED CONSENT FORM OF PATIENT INFORMED CONSENT

You are giving your consent to voluntarily participate and at your own will in this clinical research which aims to screen women for cervical cancer. Before you decide to participate in this screening project, it is important that you understand why the research is being done and how this will be conducted. Please read the following information carefully. You are free to ask anything if it is not clear or if you need more information.

You will be informed about every procedure to be performed. We will take medical history from you and we will perform a general physical examination and pervaginal examination on you.

Cervical samples will be collected from you by following standard guidelines. There are no known risks and harms associated with it. You may experience a little discomfort while cervical sample collection and may experience small amount of spotting (light vaginal bleeding) immediately or after sample collection.

You will be told about your findings in the study. Your responses to this survey will be anonymous. Please do not write any identifying information on this form. Every effort will be made by the researcher to preserve your confidentiality about your name and other contact information.

Your participation in this study is voluntary. It is up to you to decide whether or not to take part in this study. You also agree to give all relevant information needed, in full and to the best of your knowledge to the researcher. It is clarified to you that no incentive will be provided to you for participating in the study except the cost of lab investigations. If you decide to take part in this research, please sign this informed consent. After you sign the consent form, you are still free to withdraw at any point of time and without giving a reason.

You are advised to contact Dr. Ariba Nasreen on mobile number: 0333-0377791 or visit Histopathology department PNS Shifa Hospital Karachi in case of any query related to your disease.

I have been explained that laboratory investigations will be conducted to evaluate my health status and to diagnose and monitor my disease process. For this purpose, I fully agree to give my cervical sample to the researcher.

I also agree to give all relevant information needed, in full and to the best of my knowledge to the researcher. It is clarified to me that no incentive, financial assistance or reimbursement will be provided to me for participating in the study whereas I do have the right to withdraw from the study at any time.

Name of Patient: _____

Contact No. : _____

Signature of patient: _____

Date: _____

Name of Researcher: _____

Contact No. : _____

Signature of Researcher: _____

Date:_____

INFORMED CONSENT (URDU)

آپ اس کلینیکل ریسرچ میں رضاکارانہ طور پر حصہ لینے کے لیے اپنی رضامندی دے رہی ہیں جس کا مقصد خواتین کی سرد ائیکل کینسر کی اسکریننگ کرنا ہے۔ اس پروجیکٹ میں حصہ لینے سے پہلے ضروری ہے کہ آپ سمجھیں کہ یہ تحقیق کیوں کی جا رہی ہے اور کیسے کی جائے گی۔ براہ کرم درج ذیل معلومات کو غور سے پڑھیں۔ آپ کچھ بھی پوچھنے کے لیے آزاد ہیں اگر یہ واضح نہیں ہے یا اگر آپ کو مزید معلومات کی ضرورت ہے۔

آپ کو انجام دینے والے ہر طریقہ کار کے بارے میں مطلع کیا جائے گا۔ ہم آپ سے میڈیکل ہسٹری لیں گے اور ہم آپ کا عمومی جسمانی معائنہ اور پروٹیکشن معائنہ کریں گے۔ سرد ائیکل کے نمونے معیاری ہدایات پر عمل کرتے ہوئے آپ سے جمع کیا جائے گا۔ اس سے وابستہ کوئی خطرہ اور نقصانات نہیں ہیں۔ سرد ائیکل کے نمونے جمع کرنے کے دوران آپ کو تھوڑی تکلیف ہو سکتی ہے اور نمونے جمع کرنے کے بعد تھوڑی مقدار میں دھبے (ہلکے اندام نہانی سے خون بہنے) کا تجربہ ہو سکتا ہے۔

مطالعہ میں آپ کو اپنے نتائج کے بارے میں بتایا جائے گا۔ اس سروے پر آپ کے جوابات گمنام ہوں گے۔ براہ کرم اس فارم پر کوئی شناختی معلومات نہ لکھیں۔ محقق کی طرف سے آپ کے نام اور دیگر رابطہ کی معلومات کے بارے میں آپ کی رازداری کو برقرار رکھنے کی ہر ممکن کوشش کی جائے گی۔

اس مطالعہ میں آپ کی شرکت رضاکارانہ ہے۔ اس مطالعہ میں حصہ لینے یا نہ لینے کا فیصلہ کرنا آپ پر منحصر ہے۔ آپ تمام متعلقہ معلومات کو مکمل طور پر اور اپنی بہترین معلومات کے مطابق محقق کو دینے سے بھی اتفاق کرتی ہیں۔

آپ کو واضح کیا جاتا ہے کہ مطالعہ میں حصہ لینے کے لیے آپ کو لیبارٹری تحقیقات کے اخراجات کے علاوہ کوئی معاوضہ نہیں دیا جائے گا۔ اگر آپ اس تحقیق میں حصہ لینے کا فیصلہ کرتی ہیں تو براہ کرم اس باخبر رضامندی پر دستخط کریں۔ رضامندی کے فارم پر دستخط کرنے کے بعد آپ کسی بھی وقت اور وجہ بتائے بغیر دستبردار ہونے کے لیے آزاد ہیں۔ آپ کو مشورہ دیا جاتا ہے کہ ڈاکٹر اریبہ نسرین (محقق) سے موبائل نمبر:03330377791 پر رابطہ کریں یا اپنی بیماری سے متعلق کسی بھی سوال کی صورت میں ہسٹوپیتھولوجی ڈیپارٹمنٹ پی این ایس شفا ہسپتال کراچی کا دورہ کریں۔

اعلانیہ

مجھے سمجھایا گیا ہے کہ میری صحت کا جائزہ لینے اور میری بیماری کی تشخیص اور نگرانی کے لیے لیبارٹری تحقیقات کی جائیں گی۔ اس مقصد کے لیے میں محقق کو اپنا سرد ائیکل نمونہ دینے پر پوری طرح متفق ہوں اور تمام متعلقہ معلومات کو مکمل طور پر اور میری بہترین معلومات کے مطابق محقق کو دینے سے بھی اتفاق کرتی ہوں۔ مجھے واضح کیا جاتا ہے کہ مطالعہ میں حصہ لینے کے لیے کوئی مالی امداد یا معاوضہ فراہم نہیں کیا جائے گا جبکہ مجھے کسی بھی وقت مطالعہ سے دستبردار ہونے کا حق حاصل ہے۔

مریض کا نام:.....

رابطہ نمبر: .....

مریض کے دستخط / انگوٹھے کا نشان: .....

تاریخ:.....

محقق کا نام:.....

رابطہ نمبر: .....

محقق کے دستخط: .....

تاریخ:.....

E. SUBJECT EVALUATION FORM PROFORMA

Serial No: _____

MR No.: _____

Name: (optional)_____

Contact No.(optional) _____

1. **Age:**

□ 25-30 years □ 31-40 years □ 41-50 years □ 51-60 years □ 61-65 years

2. **Marital status:**

□ Married □ Widow □ Separated □Divorced

3. **Married since:**

□1-5 years □5-10 years □10-15 years □15-20 years □20+ years

4. **Ethnicity:**

□ Sindhi □ Punjabi □ Pashto □ Balochi □ Urdu speaking □ Other

5. **Education Background:**

□Uneducated □ Elementary □ Matric □ Inter □ Undergraduate □ Postgraduate □ Other

6. **Occupation:**

□ Teacher □ Health service worker □ Self-employed □ Unemployed □ Housewife □ Other

7. **Age of Menarche:**

□ 8 years □ 9years □10 years □ 11 years □ 12 years □ 12+ years

8. **No. of average menstrual cycle days**

□ less than 3 □ 4 to 5 □ 6 to 7 □ more than 7

9. **Menstrual Hygiene Product Used**

□Disposable pads □ Tampons □ Menstrual cups □ Reusable cloths

10. Average number of menstrual hygiene products used during the day

less than 3 3 to 4 5 to 6 more than 6

11. Menstrual hygiene products disposal routine

Garbage bin Throw into Toilet / Flush Bury Burn

12. History of Smoking:

Yes No

13. If yes for how long:

<1 year 1-3 years 4-5 years 5-9 years 10+ years

14. Number of cigarette smoked per day:

1 1-3 4-5 5-9 1 whole pack > 1 whole pack

15. Age at Marriage:

15-20 years 21-25 years 26-30 years 30-35 years 35+ years

16. Number of Children:

0 1 2 3 4 4+

17. History of previous Urogenital tract infection (if known):

Yes No

If Yes Specify _____

18. Miscarriage History:

Yes No

If Yes Specify _____

19. Prolong use of oral contraceptives history:

Yes No

If Yes Specify _____

20. Prior vaccination history:

Yes No

If Yes Specify _____

21. Prior screening for cervical cancer:

Yes No

22. Method of prior screening:

Clinical Examination Pap Smear Cytology Not known

23. Prior screening result:

Inadequate Sample Normal ASC-US LSIL HSIL Not known

Sign and Symptoms:

24. Bleeding between cycle:

Yes No

25. Bleeding after intercourse:

Yes No

26. Postcoital (after intercourse) pain/discomfort:

Yes No

27. Abnormal vaginal discharge:

Yes No

28. Lower abdominal pain:

Yes No

F. CYTOLOGICAL EXAMINATION

Microscopic Findings:

_____

_____

Nuclear atypia_____

Irregular nuclear shape and/or border _____

Enlarged nuclei_____

Hyperchromatic_____

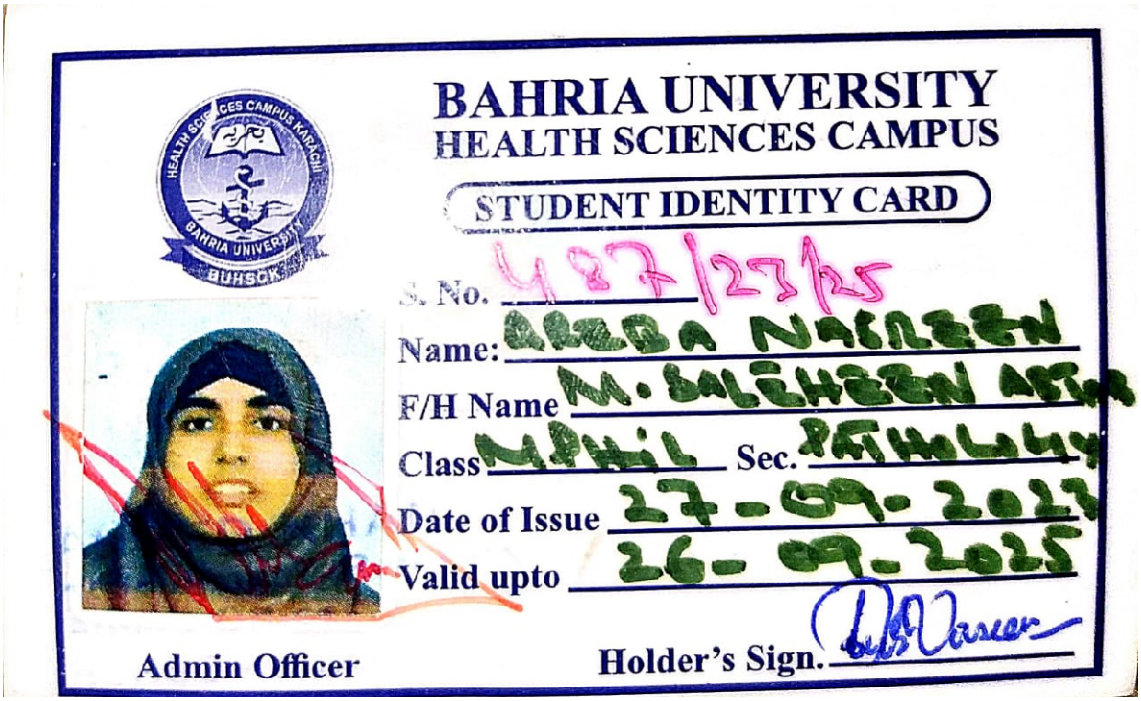
Increased N/C ratio and nuclear division_____

Tumor Cells Bethesda Grade_____

IMMUNOHISTOCHEMISTRY

LOCATION	P16	TOP2A
Cytoplasmic		
Nuclear		
Both		
Intensity Of Staining		
0		
+1		
+2		
+3		
%Age Of Positive Cells		
None		
<10%		
10-50%		
51 to 80%		
>80%		
IRS-Score		

G. HOSPITAL/INSTITUTE CARD



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