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Assessment of the applicability of p16 and top2a biomarkers for cervical cancer diagnosis in northern Tanzania

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ASSESSMENT OF THE APPLICABILITY OF p16 AND TOP2A BIOMARKERS FOR CERVICAL CANCER DIAGNOSIS IN NORTHERN TANZANIA

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Biomarkers provide valuable information for the early detection of cervical cancer. However, they are seldom utilized for the prognosis of cervical cancer in Tanzania, where visual inspection with acetic acid (VIA) or Lugol's iodine (VILI) are being utilized as the standard screening techniques. A retrospective hospital-based cross-sectional study assessing cyclin-dependent kinase inhibitor (p16) and topoisomerase II-alpha (TOP2A) proteins expression was conducted among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre, Tanzania between 1 May, 2017 to 10 May, 2018. A total of 149 studied patients (Mean age: 52.1 ± 12.9 years) were enrolled. Of these, 99 (66.4%) of women seeking care in the period under review were diagnosed with cervical cancer lesions. Moreover, only 145 cervical biopsies met the inclusion criteria for p16 and TOP2A immunohistochemistry (IHC) staining. Upon IHC staining, 103 (71.0%) and 90 (62.1%) were p16 and TOP2A positive, respectively. Histopathological class and p16/TOP2A expression levels were closely associated (Fisher's exact test, $p<0.001$). Moreover, p16/TOP2A expression levels were positively correlated with cancerous cervical lesions (Spearman's rank correlation coefficients = 0.833 and 0.687, $p = 0.006$ and 0.005, respectively). The age-adjusted odds ratio (AOR) for predicting cervical cancer lesions were independently significant for p16/TOP2A biomarkers in formalin-fixed and paraffin-embedded cervical tissues [p16: AOR=1.142 (95% confidence interval (CI): 1.059–1.232, $p<0.001$) and TOP2A: AOR=1.046 (95% CI: 1.008–1.085, $p=0.015$)]. Importantly, the diagnostic performance of p16 was higher than that of TOP2A in the diagnosis of cancerous lesions from non-cancerous cervical lesions (sensitivity: 97.2% *versus* 77.6%, accuracy: 92.8% *versus* 87.8%, respectively). Overexpression of TOP2A is linked to the grade of cervical intraepithelial neoplasia in our study, however it does not predict cervical cancer prognosis. Similarly, expression of p16 is associated with histological dysplasia grade and malignancy, suggesting its prognostic significance in the management of cervical cancerous lesions. Further bigger studies are needed to validate their applications in the early diagnosis of cervical cancer.

DECLARATION

I, Zavuga Zuberi, do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this dissertation is my original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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Candidate Name	**Signature**	**Date**

The above declaration is confirmed by

Dr. Elingarami Sauli Nkya		
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Prof. Jaffu Othniel Chilongola		
Supervisor 2	**Signature**	**Date**

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CERTIFICATION

The undersigned certifies that they have read this dissertation titled "*Assessment of the applicability of p16 and TOP2A biomarkers for cervical cancer diagnosis in Northern Tanzania*" and found the dissertation acceptable for examination for the Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology.

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Supervisor 2	**Signature**	**Date**

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DEDICATION

This work is dedicated to my parents, Zuberi A. Rumisha, and Mariam A. Mapuli, my wife, Jamila B. Duwe, and my sons, Asad and Aryan.

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LIST OF ABBREVIATIONS AND SYMBOLS

AS-CUS	Atypical Squamous Cells of Undetermined Significance
CDK	Cyclin Dependent Kinase
CIN	Cervical Intraepithelial Neoplasia
FFPE	Formalin-Fixed and Paraffin-Embedded
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HICs	High-Income Countries
hrHPV	High-Risk Human Papillomavirus
HPV	Human Papillomavirus
IHC	Immunohistochemistry
KCMC	Kilimanjaro Christian Medical Centre
KCMUCo	Kilimanjaro Christian Medical University College
KCRI	Kilimanjaro Clinical Research Institute
LMICs	Low- and Middle-Income Countries
lrHPV	Low-Risk Human Papillomavirus
Pap	Papanicolaou Test
pRb	Retinoblastoma
TOP2A	Topoisomerase-II Alpha
VIA	Visual Inspection with Acetic Acid
VILI	Visual Inspection with Lugol's Iodine

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Cervical cancer is the fourth frequently diagnosed cancer affecting women of reproductive ages globally (Bray *et al.*, 2018). According to the data from Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN), it was estimated that about 570 000 new cancer cases and 311 000 deaths worldwide in 2018 (Bray *et al.*, 2018; Ferlay *et al.*, 2019). It is considered as the prominent cause of female cancers in Tanzania, contributing to almost 9800 cancer incidence cases with approximately 6700 mortality cases in 2018 (Bruni *et al.*, 2019). This accounted for 39.0% of cancer incidence cases and 40.6% mortality cases among all women cancers, a higher rate than the average global cancer burden (Bray *et al.*, 2018).

Biomarkers expression significantly contribute in the understanding of cancer pathogenesis, early cancer detection, disease severity with an enormous potential towards access to personalized medicine (Rusling *et al.*, 2010). A number of biomarkers have been associated to the pathogenesis of cervical cancer (Brown *et al.*, 2012; Hwang & Shroyer, 2012; Mishra & Verma, 2010). However, the sensitivity and specificity of cancer detection may be improved by using a collection of biomarkers that discriminates abnormal cells from normal cells across age groups (Ding *et al.*, 2020; Dixon *et al.*, 2017; Peres *et al.*, 2016; Shi *et al.*, 2019; Sun *et al.*, 2019). Unfortunately, cyclin-dependent kinase inhibitor (p16) and topoisomerase II-alpha (TOP2A) biomarkers are not widely applied in cervical cancer diagnosis in low- and middle-income countries (LMICs) including Tanzania.

However, cell proliferation biomarkers, p16 and TOP2A play significant roles, whereby p16 protein arrests the G1/S phase by impeding the cyclin-dependent kinase (CDK) (Lambert *et al.*, 2006; Pandey *et al.*, 2018; Wu *et al.*, 2019) while TOP2A gene codes for TOP2A enzyme which is needed for relaxing DNA supercoiled structure during DNA synthesis (Del Moral-Hernández *et al.*, 2021; Dixon *et al.*, 2017; Peres *et al.*, 2016). Therefore, this study aimed to investigate the usefulness of p16 and TOP2A biomarkers in the diagnosis of dysplastic and malignant alteration of cervical epithelium and if they can be of use in predicting the prognosis of cervical carcinogenesis in Tanzania.

1.2 Statement of the Problem

Cervical cancer is the fourth frequently diagnosed cancer that affects women at their reproductive ages globally, with more than 265 000 deaths occurring in LMICs (Bray *et al.*, 2018; Cohen *et al.*, 2019; Ferlay *et al.*, 2019). In Tanzania, the 2018 cervical cancer burden was estimated at 22.4% and 32.7% for the crude mortality and crude incidence rates, respectively (Bruni *et al.*, 2019). Many high-income countries (HICs) have successfully reduced incidence and mortality rates of cervical cancer by 80% due to effective population-based early detection and screening programs that are well organized and with high coverage rates. However, cervical cancer early diagnosis programs have not been able to control the disease in low-resource countries like Tanzania, partly because of lack, or limited access and low performances of available methods (Bray *et al.*, 2018; Ferlay *et al.*, 2019).

Currently, cervical cancer screening techniques in Tanzania primarily rely on visual inspection with acetic acid (VIA) or visual inspection with Lugol's iodine (VILI) as the standard screening approach (Bruni *et al.*, 2019; Dartell *et al.*, 2014), which is freely available at various levels of care in several governmental and faith-based facilities. Although VIA/VILI has lower sensitivity and specificity compared to *Papanicolaou* ('Pap') test and Human papillomavirus (HPV) DNA testing (Dartell *et al.*, 2014; Ngoma *et al.*, 2010), it is still used in several LMICs due to its low-cost, single visitation strategy and a high burden of cervical cancer in LMICs. However, Pap test is available in the zonal hospitals in Tanzania.

Several cancer biomarkers, especially when applied individually, may not detect cervical cancer as it develops, making them unsuitable for screening and diagnosis of cervical cancer (Brown *et al.*, 2012; Sun *et al.*, 2019; Tornesello *et al.*, 2013). However, a combination of biomarkers may improve the sensitivity and specificity of cancer detection in discriminating normal cells from abnormal cells across age groups (Ding *et al.*, 2020; Dixon *et al.*, 2017; Peres *et al.*, 2016; Shi *et al.*, 2019; Sun *et al.*, 2019). During pathogenesis of cervical cancer, the expression of E7 viral oncoprotein inactivates retinoblastoma protein, which consequently increases the E2 transcription factor (E2F) in the cell, which leads to an increased p16. Likewise, the TOP2A gene encodes for DNA topoisomerase, a nucleic enzyme responsible for unwinding DNA supercoiled during DNA synthesis. Expression levels of p16 and TOP2A have been reported as biomarkers in cervical cancer diagnosis in high-resource countries (Ding *et al.*, 2020; Dixon *et al.*, 2017; Peres *et al.*, 2016), which necessitates the need for their feasibility study in the setting of LMICs like Tanzania.

These cellular biomarkers (p16 and TOP2A) are emerging novel biomarkers for the early diagnosis and prognosis of cervical cancer (Sahasrabuddhe *et al.*, 2011; Tornesello *et al.*, 2013). Moreover, the expression levels of p16 and TOP2A proteins have been associated with the progression of cancerous lesions and improving the accuracy of the clinico-histopathological diagnosis of cervical lesions (Cunningham *et al.*, 2015; Del Moral-Hernández *et al.*, 2021; Moshi *et al.*, 2018; Ngoma *et al.*, 2010; Shi *et al.*, 2019). This may contribute to insufficient data being reported on the prevalence of cervical cancer in Tanzania. Likewise, low-cost, sensitive and specific screening methods including low-cost HPV DNA tests such as *care*HPV for detection of high-risk HPV (hrHPV) genotypes are highly needed in Tanzania (Katanga *et al.*, 2019). Therefore, this study was conceived to assess the applicability of p16 and TOP2A biomarkers for cervical cancer diagnosis in Northern Tanzania.

1.3 Rationale of the Study

The rationale of this study was to evaluate the utility of p16 and TOP2A as potential biomarkers in dysplastic and malignant alteration of cervical epithelium by analyzing a series of benign, precancerous and cancerous cervical lesions among women seeking cervical cancer care at tertiary referral hospital. This would lead to p16 and TOP2A expression levels which might be of any use in predicting prognosis in cervical carcinogenesis in Tanzania.

1.4 Research Objectives

1.4.1 Main Objective

To assess the applicability of p16 and TOP2A biomarkers for cervical cancer diagnosis in Northern Tanzania.

1.4.2 Specific Objectives

(i) To associate histopathological classification and patient's clinical information among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018.

(ii) To assess p16 and TOP2A protein expressions by immunohistochemistry using cervical biopsies from women seeking care at KCMC from 1 May, 2017 to 10 May, 2018.

1.5 Research Questions

(i) Are the expression levels of p16 and/or TOP2A biomarkers significantly associated with clinico-histopathological characteristics in the diagnosis of cervical cancer among women seeking care at KCMC?

(ii) What is the association between histopathological classification and patient's clinical information among women seeking cervical cancer care at KCMC from 1 May, 2017 to 10 May, 2018

1.6 Significance of the Study

This study was conducted to understand the association between p16 and TOP2A biomarkers, and clinico-histopathological characteristics of cervical lesions among women seeking cervical cancer care at KCMC. The study findings were useful in improving the diagnosis of dysplastic and malignant alteration of cervical epithelium by analyzing a series of benign, precancerous and cancerous cervical lesions.

1.7 Delineation of the Study

The focus of this study is to assess the applicability of p16 and TOP2A biomarkers for cervical cancer diagnosis in Northern Tanzania. This study included a relatively low number of analyzed archived cervical biopsies which could be contributed by missing patients' data based on the retrospective study design. In addition, this study did not identify and genotype HPV subtypes due to the limited availability of research funds. However, HPV genotyping helps to assess the genetic instability of oncogenic HPV subtypes towards infecting normal cells, which could eventually predict the risks of cervical cancer and its precursors' development in association with the expression of p16 and TOP2A biomarkers in the studied populations, and Tanzania population at large. Yet, the study findings from the associations between p16 and TOP2A biomarkers with clinico-histopathological features may be useful in classifying cervical lesions among women seeking cervical cancer care in tertiary hospitals. Moreover, lack of data on the histopathological differential diagnosis of endocervical polyps, squamous cell carcinoma and adenocarcinoma possibly contributed to the observed differences in the immunoexpressions of p16 and TOP2A biomarkers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Cervical Cancer

Cervical cancer is a slow-growing disease that arises from the cervical cells, with chronic HPV infection causing at least 95% of cervical cancer cases (Small *et al.*, 2017). Cervical cancer development involves a gradual advancement from normal cervical epithelium tissue to pre-cancerous cervical lesion followed by the cervical intraepithelial lesion (CIN) which if persists results in invasive cervical cancer lesions (Fig. 1) (Crow, 2012; Schiffman *et al.*, 2016).

Cervical cancer is mainly caused by persistent HPV infection (McGraw & Ferrante, 2014). However, practicing sexual intercourse at early age <18 years, high parity, excessive alcohol consumption, cigarette smoking, early pregnancy at <18 years of age, multiple sex partners, prolonged use of contraceptives, marital status, abortion, and co-infection with sexually transmitted infections are considered as the risk factors for cervical cancer (Cohen *et al.*, 2019; Small *et al.*, 2017). Cervical cancer patients are clinically asymptomatic in the early stages with underlying symptoms, including abnormal vaginal bleeding, post-coital pain or bleeding, abnormal vaginal discharge, and lower abdominal pain (Cohen *et al.*, 2019).

An effective way for preventing cervical cancer is through the detection of cervical cancer in its early stages (McGraw & Ferrante, 2014). A Pap test is widely used to identify invasive pre-cancerous cervical lesions and early stages of cervical cancer and has considerably reduced the cervical cancer burden in HICs (Ferlay *et al.*, 2019; McGraw & Ferrante, 2014). However, cervical cancer screening programs have been unable to control the disease in LMICs like Tanzania, partly because of lack, or limited access and low performances of available methods (Bray *et al.*, 2018; Ferlay *et al.*, 2019). The most used cervical cancer screening methods in Tanzania are VIA and/or VILI assays and Pap tests (Ngoma *et al.*, 2010).

The HPV vaccination can prevent cervical cancer by preventing hrHPV subtypes and their subsequent cervical pathogenesis (Cohen *et al.*, 2019). Gardasil® and Cervarix® are the two HPV vaccines approved in more than 100 countries worldwide (McGraw & Ferrante, 2014; Small *et al.*, 2017). Gardasil® is a recombinant vaccine against HPV-6, 11, 16 and 18 subtypes (quadrivalent) while Cervarix® targets HPV-16 and 18 subtypes (bivalent) (Cohen *et al.*, 2019; McGraw & Ferrante, 2014; Small *et al.*, 2017).

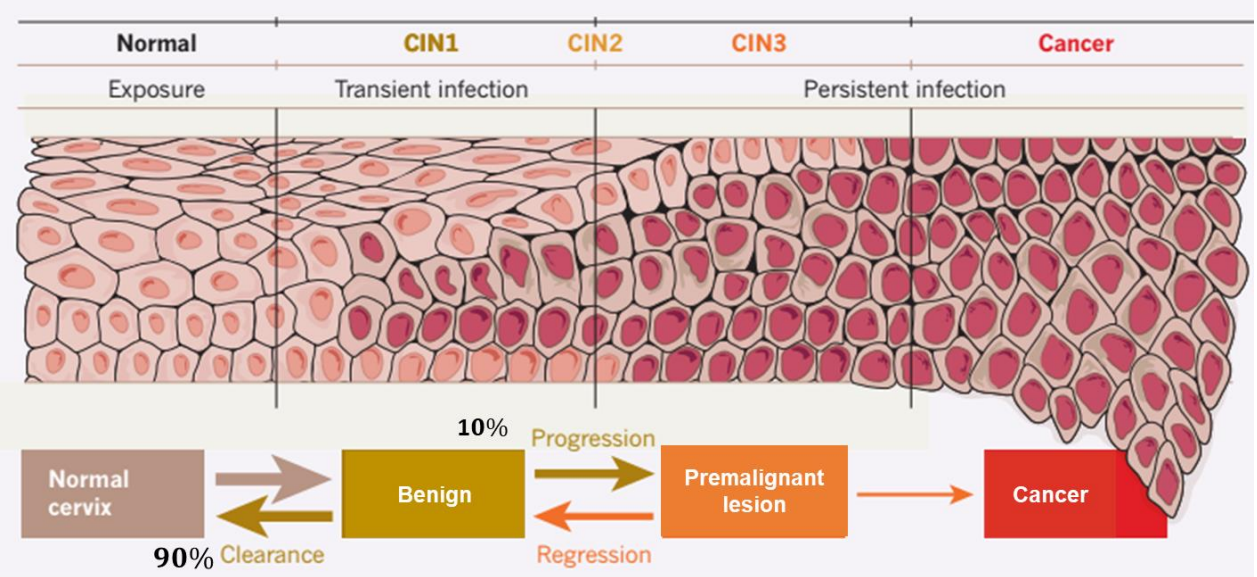

Figure 1: Progression of cervical cancer (Crow, 2012)

2.2 The Burden of Cervical Cancer

Worldwide, cervical cancer accounted for about 570 000 new cases with more than 311 500 cervical cancer-related deaths in the year 2018 (Bray *et al.*, 2018). It is estimated that nearly 80% of the new cervical cancer cases and 85% of the cancer-related deaths occurred in LMICs within the same year (Cohen *et al.*, 2019; Ferlay *et al.*, 2019). Cervical cancer is considered of major public health concern by affecting women at their reproductive age especially in LMICs. However, HICs have made remarkable progress in reducing cervical cancer incidence and mortality rates relative to the LMICs (Beddoe, 2019; Black & Richmond, 2018). This has been attributed to effective population-based early detection and screening programs that are well-organized and with high-coverage rates (Beddoe, 2019).

According to the GLOBOCAN data, cervical cancer accounted for about 110 345 new cases with 72 712 cervical cancer-related deaths in sub-Saharan Africa in 2020 (International Agency for Research on cancer, 2021). In contrast, East Africa has the highest rates of cervical cancer of 40.1 and 20.6 per 100 000 women with age-standardized incidence and mortality rates, respectively (Sung *et al.*, 2021). Moreover, cervical cancer is the leading cause of female cancers in Tanzania, where 10 241 new cervical cancer cases (age-standardized incidence rate of 34.3 per 100 000 women) and 6525 cervical cancer-related deaths (age-standardized mortality rate of 21.8 per 100 000 women) were reported in 2020 by the GLOBOCAN data (Sung *et al.*, 2021). These reported data point-out the importance of an effective cervical cancer screening program.

2.3 Human Papillomavirus and Pathogenesis of Cervical Cancer

As the major risk factor for cervical carcinogenesis, HPV plays a critical role (Cohen *et al.*, 2019; Crow, 2012). The HPV is a naked, double-stranded DNA virus with roughly 8000 base pairs that belongs to the *Papillomaviridae* (Dias *et al.*, 2020). It also has nine open reading frames, including six early genes (E1, E2, E4, E5, E6, and E7), as well as two late genes (L1 and L2). However, a non-coding large control region regulates other HPV genes (Brown *et al.*, 2012; Schiffman *et al.*, 2016). About forty HPV subtypes are linked to cervical cancer progression, and they are further divided into low-risk (lrHPV) and high-risk (hrHPV) subtypes (Crow, 2012).

Moreover, the most common lrHPV subtypes are HPV-6 and -11 which are often associated with genital warts whereas the hrHPV subtypes are HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 which are associated with the occurrence of cervical cancer (Crow, 2012; Dias *et al.*, 2020). The hrHPV-16 and 18 subtypes are responsible for about 75% of cervical cancer cases (Crow, 2012; Small *et al.*, 2017). Approximately 90% of the HPV infections are transitory which resolve on their own within two years (McGraw & Ferrante, 2014; Small *et al.*, 2017). The remaining 10% of the HPV infections can persist, leading to the development of pre-cancerous cervical lesions (CIN or adenocarcinoma *in situ*) which can occur in less than 1 year (Small *et al.*, 2017).

The incorporation of HPV DNA into the host cells is connected to the progression of pre-cancerous cervical lesions to invasive cervical cancer (Schiffman *et al.*, 2016). During cervical cancer pathogenesis, the expression of E7 viral oncoprotein inactivates pRb, which consequently increases the E2F activation factors in the cell, which leads to an increased overexpression of CDK inhibitors in the S-phase of the cell cycle, which plays a vital role for the cervical cancer initiation and its progression (Ibeanu, 2011; Schiffman *et al.*, 2016).

2.4 Current Techniques for Screening of Cervical Cancer

The screening procedures for cervical cancer involve a combination of studying cervical cells and testing for the presence of HPV and if possible their related subtypes (Denny, 2015). However, visual inspection-based assays (VIA and/or VILI) re-emerged as the simple, fast, reliable and economical screening methods in low-resource countries regardless of their poor sensitivity (Ardahan & Temel, 2011; Denny, 2015; Sankaranarayanan *et al.*, 2012). Visual inspection-based assays have been recommended for cervical screening women aged 30-59 years old in LIMCs (Sankaranarayanan *et al.*, 2012).

Moreover, VIA entails an eye examination of the cervix under the illumination of a lamp to identify acetowhite lesions, followed by swabbing or spraying diluted acetic acid, generally 3-5%, to the cervix (Qureshi *et al.*, 2010; Sankaranarayanan *et al.*, 2012). The lack of acetowhite lesions in the squamocolumnar junction (SJC) is a negative test, while opaque acetowhite lesions in the SJC of the cervix is a positive test (Qureshi *et al.*, 2010; Sankaranarayanan *et al.*, 2012). Unfortunately, only 4-55% of examined women in many trials using the VIA test for cervical cancer screening had positive findings (Ardahan & Temel, 2011).

Similarly, VILI involves direct eye examination of the cervix following the administration of 5% Lugol's iodine to detect mustard-yellowish lesions in the SJC of the cervix (Sankaranarayanan *et al.*, 2012). A negative test for VILI is characterized by the formation of mahogany brown or black in the squamous epithelium without color change in the SJC of the cervix (Qureshi *et al.*, 2010).

Pap test is an effective method that is widely used for cervical cancer screening especially in HICs. Due to the wide-coverage of population-based early screening of cervical cancer and the sensitivity of the Pap test in detecting high-grade squamous intraepithelial lesion, it dramatically decreased cervical cancer incidence and death rates by almost 80% in HICs (McGraw & Ferrante, 2014; Sachan *et al.*, 2018). A Pap test can be performed as a conventional or liquid-based pap test. Cervical cells are immediately spread on a microscope slide in the former approach, but in the liquid-based pap test, the obtained cells are put in a tiny glass vial containing preserving liquid (McGraw & Ferrante, 2014; Sherwani *et al.*, 2007). After staining and microscopic examinations, the cytological specimens are categorized based on the loss amount of cytoplasmic maturity, aberrant mitotic figures, and changes in nuclear size and shape of the cervical cells (Table 1). There are several ongoing debates worldwide about which way is superior than another. However, studies have shown that, no statistically significant variations exist in terms of specificity and sensitivity of the conventional and liquid-based cytology pap smear test (Sherwani *et al.*, 2007; Sigurdsson, 2013).

Although pap smear tests have reduced the incidence of cervical cancer in HICs, they have been linked to a high rate of false-positive results, prompting the development of molecular tests for HPV detection, such as hybrid capture II (HC2) (Qiagen, Hilden, Germany), *care*HPV (Qiagen, Gaithersburg, MD, USA), and real-time Polymerase Chain Reaction assays such as Cobas® 4800. (e.g., Illumina platforms, etc.) (Dias *et al.*, 2020; McGraw & Ferrante, 2014). The HPV genotyping is an effective tool for cervical cancer screening in clinical settings. The first FDA-approved technique for HPV genotyping in clinical settings was the Cobas® 4800 HPV which can detect 14 hrHPV including HPV-16 and -18. The HC2 can detect 13 hrHPV as a triage test

for women with atypical squamous cells of undetermined significance (AS-CUS) in combination with studying cervical cells.

Table 1: Most common classification systems for cervical lesions

Histology		Cytology			Molecular
CIN	**LAST**	**Pap**	**WHO**	**Bethesda**	
Normal	Normal	I	Negative	NILM	Normal cervix
		II	Squamous atypia	AS-CUS	
CIN1	LSIL	III IIID	Mild	LSIL	HPV infection
CIN2	HSIL		Moderate	HSIL	Pre-cancer
CIN3		IV	Severe		
Cancer	Cancer	V	Cancer	Cancer	Cancer

Schiffman *et al.*(2016)

AS-CUS atypical squamous cells of undetermined significance
NILM negative for intraepithelial lesion and malignancy
HSIL high-grade squamous intraepithelial lesions.
CIN cervical intraepithelial neoplasia
LAST lower anogenital squamous terminology
LSIL low-grade squamous intraepithelial lesions

2.5 Roles of Studied Biomarkers in the Diagnosis of Cervical Cancer

The working group and the biomarkers consortium of the US National Institute of Health defines a biomarker as "a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention" (Mishra & Verma, 2010). Several biomarkers for cervical cancer detection in cytological and histological samples have been discovered. The majority of biomarkers are involved in the control and proliferation of cell cycle, transduction pathways, and DNA synthesis (Brown *et al.*, 2012; Dasari *et al.*, 2015; Mishra & Verma, 2010).

Several immunoassays including but not limited to immunohistochemistry (IHC), enzyme-linked immunosorbent assay, lateral flow immunoassays, and immunoblots such as western blots can be used for routine cervical cancer screening (Güzel *et al.*, 2021). However, IHC is the predominant immunoassay for cervical cancer diagnosis in a clinical setting, because it is well-established and stains are readily available, frozen or archived samples can be used, fast turn-around time, and a relatively low risk of infectious agents to humans (Duraiyan *et al.*, 2012; Güzel *et al.*, 2021). The drawbacks include expensive IHC equipment, the technique being prone to human errors resulting to inter-observer variability of the results, and complexities in the quantification of results (De Matos *et al.*, 2010).

A nuclear and cytoplasmic protein, p16 may help in the screening, diagnosis and prognosis of dysplastic cervical cells with persistent hrHPV infection (Lin *et al.*, 2014; Volgareva *et al.*, 2004). The p16 is coded by the tumor suppressor gene *INK4a* which has a significant function in the regulation of the CDK-Rb-E2F pathway (Volgareva *et al.*, 2004; Wu *et al.*, 2019). Usually, cell cycle activation depends on the binding of pRb to E2F transcription factors. However, in HPV transformed cells, E7 oncogene inhibits the binding of pRb protein to E2F transcription factors which inactivate CDK-4/6, leading to increased expression p16 with arresting of G1-S phase (Brown *et al.*, 2012; Volgareva *et al.*, 2004). In addition, the diagnostic utility of p16 expression as an individual biomarker has been reported in differentiating cancerous cervical lesions from benign and precancerous cervical lesions in HICs (Gustinucci *et al.*, 2012; Marcus *et al.*, 2017) and LMICs (Volgareva *et al.*, 2004; Wu *et al.*, 2019; Zhang & Shen, 2018). In Tanzania, limited information exists about the applicability of p16 immunostaining in the diagnosis of cervical cancer lesions. Nevertheless, p16/Ki-67 dual-staining histology, increased sensitivity of CIN lesion diagnosis and outcome prediction of CIN-2 in patients with hrHPV-16 and -58 were observed (Li *et al.*, 2019). Similarly, p16/Ki-67 dual-staining cytology was an excellent triage test for HPV-positive women in Western Kenya although faced low sensitivity (Orang'o *et al.*, 2020). In addition, p16/Ki-67 dual-staining histology was not associated with CIN grade on follow-up excisional pathology (Marcus *et al.*, 2017). This suggests the feasibility of combining p16 and TOP2A biomarkers to assess their potential diagnostic performance for cervical cancer lesions in Tanzania.

A nuclear protein gene, TOP2A encodes for DNA topoisomerase, a nucleic enzyme responsible for unwinding the supercoiled DNA strands during DNA synthesis (Del Moral-Hernández *et al.*, 2021; Dixon *et al.*, 2017; Peres *et al.*, 2016). The TOP2A protein overexpression has been associated with progression from CIN-2/3 to cervical cancer lesions. However, very limited findings have been reported assessing the expression of TOP2A protein using archived cervical biopsies in HICs and LMICs. High-expression of TOP2A protein has been observed in the cervical cytology samples infected with HPV-6, 11, and 18 (Peres *et al.*, 2016). Similarly, the TO2A biomarker has been demonstrated as the best biomarker in discriminating high-grade squamous intraepithelial lesions (HSIL) from low-grade squamous intraepithelial lesions (LSIL) in liquid-based cytology samples (Del Moral-Hernández *et al.*, 2021). In consideration of the highly diversified genetic populations in Tanzania, the expression levels of p16 and TOP2A biomarkers for cervical cancer diagnosis could produce different patterns relative to that of HICs, which might improve the diagnosis of cervical cancer in Tanzania.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

A retrospective cross-sectional study was deployed using archived cervical tissue blocks obtained from women seeking cervical cancer care at KCMC, Northern Tanzania from 1 May, 2017 to 10 May, 2018.

3.2 Sample Size Estimation

The study samples were retrospectively collected from 1 May, 2017 to 10 May, 2018 with a sample size minimally estimated at 343 for cervical biopsies calculated by the formula:

$$N = \frac{Z^2 P\,(100 - P)}{d^2}$$

Where; N denotes the minimum sample size, P indicates the prevalence of cervical cancer in Tanzania = 32.7% (Bruni *et al.*, 2019), d is the estimate precision = 5% and; Z represents the standard value at 95% confidence interval = 1.96.

3.3 Retrieval of Study Samples and Related Clinical Information

Patients' clinical and pathological information, hematoxylin and eosin (H&E) slides and formalin-fixed and paraffin-embedded (FFPE) cervical tissue blocks were all retrieved for review and diagnosis confirmation. Information including the age of the patients, date of examination, clinical presentations of the patients, clinical diagnosis and microscopic findings of samples were collected.

The inclusion criteria involved patients with complete clinico-histopathological features and good morphology of their cervical biopsies. The exclusion criteria involved missing tissue block and incomplete clinical histopathological information.

The H&E slides were reviewed with assistance from a well-trained anatomical pathologist. In case of lost or broken slides, the sections were re-cut from the tissue blocks and stained with the H&E staining technique. The H&E stained slides were microscopically examined at 40x, 100x and 200x for histopathological features and classification of the cervical cancer cases according to the World Health Organization classification of tumors from women reproductive organs

(Kurman *et al.*, 2014). Additionally, epithelial cells were classified into how much the epithelial cells were affected into a sub-class 'precancerous cervical lesion' having three-tiered categories: CIN-1, -2 and -3 (Table 1). Moreover, benign cervical lesion was further classified into cervicitis, endocervical polyps, and others including nabothian cyst, cervical koilocytosis and cervical papilloma.

3.4 Assessment of p16 and TOP2A Biomarkers by Immunohistochemistry

3.4.1 Tissue Sectioning and Preparations

From all the FFPE tissue blocks that met inclusion criteria, two slides (labelled p16 and TOP2A) were sectioned at 3 μm and placed on the DFrost Plus positively charged slides (Diapath S.P.A., Martinengo BG, Italy). Tissue sections were submerged into the floating water bath at 45 °C to remove creases and alterations. Tissue bonding was achieved by baking the sections in a hot air oven at 40 °C overnight in removing embedded wax before p16 and TOP2A IHC staining. The slides with tissue sections were next day deparaffinized using two changes of xylene for 8 minutes each. The slides were thereafter rehydrated using decreasing grades of ethanol solution (100%, 95%, 80%, and 70%, respectively) with 10 dips in each solution.

3.4.2 Immunohistochemistry Staining

(i) Materials and Reagents

Monoclonal pre-diluted primary antibody p16 clone G175-405 (Medaysis Company, Livermore, CA, USA) and TOP2A clone Ki-S1 pre-diluted primary antibody (Medaysis Company, Livermore, CA, USA). The high pH EnVision™ FLEX Mini Kit (Dako Denmark A/S, Glostrup, Denmark) consisted of target retrieval solution high pH (50x), peroxidase-blocking reagent, DAB+ Chromogen, horseradish peroxidase (HRP), substrate buffer and wash buffer (20x). Other materials and reagents included: Absolute ethanol, xylene, moisture chamber, DFrost Plus positively charged slides (Diapath S.P.A., Martinengo BG, Italy), hydrophobic pen (Dako Denmark A/S, Glostrup, Denmark) and pressure cooker (Kanchan International Ltd., Mumbai, India).

(ii) Procedures

All the procedures were performed in the humidity chamber to avoid drying up of slides between the steps. During IHC staining, tissue sections were encircled with a hydrophobic pen (Dako

Denmark A/S, Glostrup, Denmark) and the endogenous peroxide activity was blocked using a peroxidase-blocking reagent, ready-to-use (Dako Denmark A/S, Glostrup, Denmark) for 15 minutes. Slides were rinsed with distilled water for 3 minutes after blocking endogenous peroxidase, then antigen retrieval was performed by pouring 500 mL of tap water into a pressure cooker (Kanchan International Ltd., Mumbai, India) and pre-heated to 65 °C for 10 minutes. Cold citrate buffer pH 6.0 was added into a small plastic container dish and a slide rack with slides placed into the dish. Thereafter, a small plastic container was placed into a pressure cooker with the lid being tightened. Then the tap water was heated until it produced steam in the citrate buffer retrieval solution. After the pressure cooker reached maximum pressure (approximately 15 minutes), it was left to cool for 10 minutes at room temperature. Then, a plastic dish container was removed from the pressure cooker and the retrieval solution poured out. Slides were allowed to cool using tap water for another 10 minutes then rinsed with diluted wash buffer (20x) for 5 minutes.

All sections were incubated with pre-diluted primary antibody p16 clone G175-405 (Medaysis Company, Livermore, CA, USA) and TOP2A clone Ki-S1 pre-diluted primary antibody (Medaysis Company, Livermore, CA, USA), respectively for 30 minutes. This was then followed with washing with wash buffer (20x) for 5 minutes, followed by incubation with universal horseradish peroxidase (HRP) for 30 minutes and washing with phosphate-buffered saline (PBS) reagent twice each for 3 minutes. Moreover, sections were then incubated with 3, 3' diaminobenzidine (DAB) + Chromogen (Dako Denmark A/S, Glostrup, Denmark) for 10 minutes and then rinsed by water for 2 minutes.

Thereafter, slides were counterstained with 17 dips in hematoxylin and bluing for 5 minutes. Sections were dehydrated in the ascending grades of ethanol solution (70%, 80%, 95%, and 100%, respectively), and then cleared in two changes of xylene for 5 minutes each. Finally, the sections were covered using mounting medium by using Tissue-Tek® Coverslipper (Sakura Finetek Inc., Torrance, CA, USA). In reducing false-positive results, positive controls cervical squamous cell carcinoma tissue for p16, and breast carcinoma tissue for TOP2A were used.

3.5 Immunohistochemistry Assessment

The tissue sections were microscopically evaluated by counting the number of proliferating cells and the intensity of positively stained cells for p16 and TOP2A. Positive cells for p16 were given an immunoscore of 1 to 2+ to indicate high cell proliferation or high intensity while an immunoscore of 0 was given to indicate the absence of cell proliferation or low intensity. For

positive TOP2A cells, an immunoscore of 1–2+ was given to indicate moderate cell proliferation or moderate intensity, an immunoscore of 3+ was given to indicate high cell proliferation or high intensity while an immunoscore of 0 indicated the absence of cell proliferation or low intensity.

3.6 Data management and Statistical Analysis

Raw data for histopathological features and classification as well as biomarkers expression data were entered into the Microsoft® Excel 2013 and exported to R software version 3.4.4 and 4.0.2 (https://www.r-project.org) for determination of their statistical significances as presented in tables and figures. Descriptive statistics such as mean, frequency and standard deviation were used to summarize numerical data. The associations between clinico-histopathological information and biomarkers expression were performed using Fisher's exact test. Moreover, a logistic regression model was used to estimate relationships between the expression of p16 and TOP2A biomarkers with the development of cervical cancer lesions by estimating age-adjusted odds ratio (OR) with 95% confidence intervals (CIs). In addition, the performance of p16 and TOP2A biomarkers was evaluated by assessing their sensitivity, specificity, and accuracy in differentiating cancerous cervical lesions from benign and precancerous cervical lesions. Furthermore, Spearman's rank correlation test was used to assess the strength of relationships between age, histopathological factors, and biomarkers expression. The results with two-sided $p<0.05$ were considered statistically significant.

3.7 Ethical Considerations

The National Health Research Ethics Committee (NatHREC) of Tanzania's National Institute for Medical Research granted ethical approval with permission number NIMR/HQ/R.8a/Vol. IX/2764. Before starting the study, a letter was submitted to KCMC seeking collaboration, and the Pathology department gave permission. Written informed consent was not applicable due to the retrospective design of this study.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Summary Statistics of Retrieved Cervical Biopsies

A total of 149 cervical biopsies were retrieved from KCMC between 1 May, 2017 and 10 May, 2018. The patients' age was from 23 to 83 years, with a mean and standard deviation of 52.1±12.9 years old, respectively. About 33.6% of women with histopathological samples were predominantly in the age group between 41 and 50 years old (Table 2).

Table 2: Clinico-histopathological features among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre between 1 May, 2017 and 10 May, 2018

Characteristic	N (%)
Patients' age (years)	
≤ 30	5 (3.3)
31 – 40	22 (14.8)
41 – 50	50 (33.6)
51 – 60	34 (22.8)
61 – 70	25 (16.8)
> 70	13 (8.7)
Total	149 (100)
Type of cervical lesion	
Benign cervical lesion	33 (22.2)
Pre-cancerous cervical lesion	11 (7.4)
Cancerous cervical lesion	99 (66.4)
Non-definitive diagnosis	6 (4.0)
Total	149 (100)
Clinical symptoms	
Per vaginal bleeding	64 (42.9)
Vaginal discharge	43 (28.9)
Lower abdominal pain	35 (23.5)
Others	7 (4.7)
Total	149 (100)
Biomarkers expression	
p16	
Low	42 (29.0)
High	103 (71.0)
Total	145 (100)
TOP2A	
Low	55 (37.9)
Moderate	29 (20.0)
High	61 (42.1)
Total	**145 (100)**

4.2 Histopathological Classification of Retrieved Cervical Biopsies

4.2.1 The Burden of Cervical Disease Based on Retrieved Cervical Biopsies

Of 149 cervical biopsies examined in the study period, only 6 (4%) were reported to have non-definitive diagnoses while the majority showed pathologic abnormalities ranging from cervicitis to cervical cancer lesions. The KCMC cancer registry recorded 419 cervical cancer cases between 2013 and 2018 in the study area. However, through this study, 99 (66.4%) of women seeking care in the period under review were diagnosed with cervical cancer lesions, while benign and pre-cancerous cervical lesions accounted for 33 (22.2%) and 11 (7.4%), respectively (Table 2).

Cervical cancer is the most prevalent cancer affecting Tanzanian women (Bruni *et al.*, 2019), and it was also the leading cervical disease in this study, accounting for 66.4% among women seeking care at KCMC between 1 May, 2017 to 10 May, 2018. This study shows that 7.4% of cases were pre-cancerous cervical lesions, indicating that the magnitudes of pre-cancerous cervical lesions were far below compared to cancerous cervical lesions. This difference might be due to potential reasons, such as less awareness on the importance of cervical cancer screening among the high-risk women aged ≥15 years of age, women's attitudes towards fear and physical discomfort related to the screening process, and also limited access to the cervical cancer screening facilities (Cunningham *et al.*, 2015; Lyimo & Beran, 2012; Moshi *et al.*, 2018).

4.2.2 Distribution of Cervical Lesions by Age Categories

The age group above 70 years old accounted for 86.4 % of cervical cancer incidences among women seeking care, whereas the age group ≤ 30 years old accounted for the smallest percentage. In contrast, the age group ≤ 30 had a high prevalence of benign cervical lesions, whereas the older age had a low rate. However, the age group ≤ 30 had the largest number of pre-cancerous cases, accounting for just 20% of all cervical cases in the age group (Table 3).

Table 3: Distribution of cervical lesions among women seeking care at Kilimanjaro Christian Medical Centre by age group from 1 May, 2017 to 10 May, 2018

Patients' age (years)	Benign cervical lesion n (%)	Pre-cancerous cervical lesion n (%)	Cancerous cervical lesion n (%)	Non-definitive diagnosis n (%)	Total N (%)
≤ 30	2 (40.0)	1 (20.0)	2 (40.0)	-	5 (100)
31 – 40	6 (27.3)	3 (13.6)	12 (54.5)	1 (4.5)	22 (100)
41 – 50	13 (26.0)	6 (12.0)	29 (58.0)	2 (4.0)	50 (100)
51 – 60	6 (17.6)	-	26 (76.5)	2 (5.9)	34 (100)
61 – 70	5 (20.0)	1 (4.0)	19 (76.0)	-	25 (100)
> 70	1 (7.7)	-	11 (86.4)	1 (7.7)	13 (100)
Total	**33 (22.2)**	**11 (7.4)**	**99 (66.4)**	**6 (4.0)**	**149 (100)**

$\chi^2 = 15.43$, $p=0.4207$

The magnitude of cervical cancer in post-menopausal (aged >50 years of age) women in this study were about 78% among the women seeking care. A slightly similar finding was reported in the study conducted in Hawassa district, Southern Ethiopia that had 72% of the post-menopausal cervical cancer among symptomatic women (Ameya & Yerakly, 2017). Conceivably reason behind these marginally similar findings might be limited knowledge on the importance of cervical cancer screening and/or ignorance on the importance of immediate investigation following post-menopausal bleeding (Ergete & Tesfaye, 2001).

The age group between 41 and 50 years old recorded the highest proportion of cervical cases across all types of cervical lesions. There was a steady rise in the proportion of cervical cases across all types of cervical lesions in the age groups from ≤30 to 41–50 years old. The decline of cervical cases across all type of cervical lesions dominated age groups from 41–50 to >70 years of age. In contrast, there was a slight difference in the fraction of benign cervical lesions and pre-cancerous cervical lesions across all age groups as seen in Fig. 2.

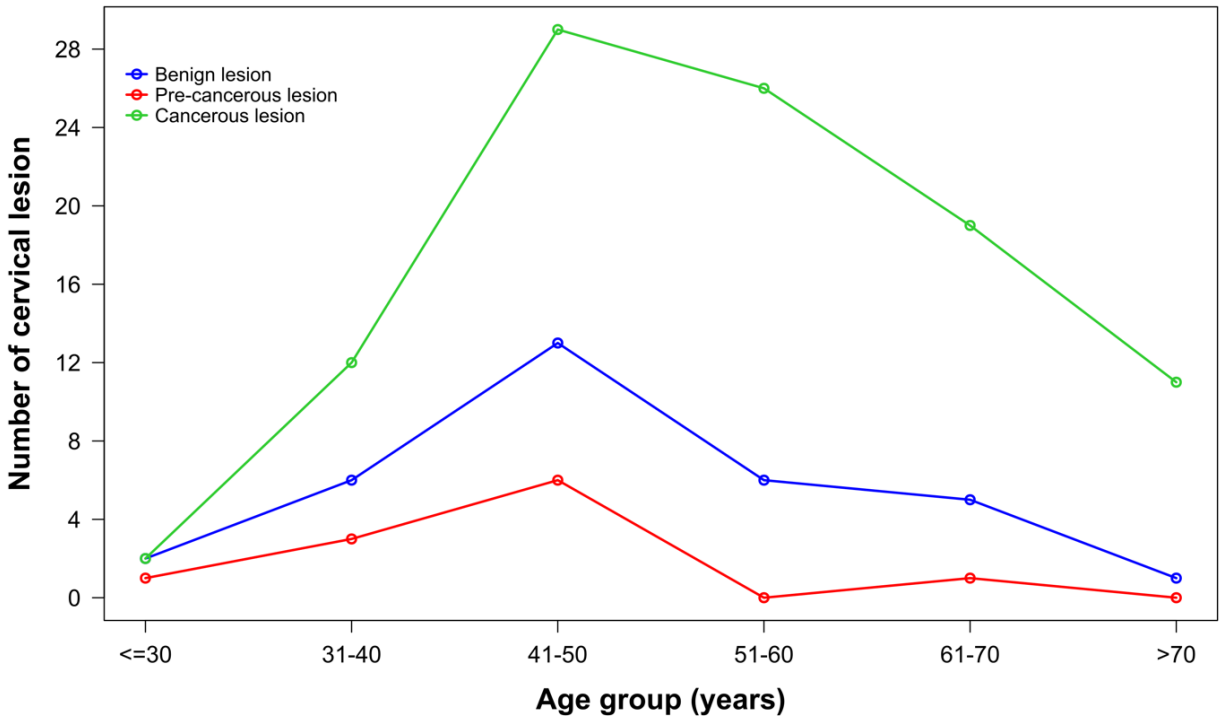

Figure 2: Trend of cervical lesion among women seeking care at Kilimanjaro Christian Medical Centre by age group from 1 May, 2017 to 10 May, 2018

The magnitude of cervical lesions increased across all lesion types among age groups between ≤30 and 41–50 years of age, although there was no statistical significance between the type of cervical lesion against patients' age (p=0.4207). This may be due to the high risk of exposure to cervical cancer among the Tanzanian female population aged ≥15 years of age (Bruni *et al.*, 2019). However, a continual decrease in the number of cervical lesions between 41–50 and >70 years of age may be linked to the higher chance of being screened at an older age, especially in urban areas for the age groups between 40–49 and >50 years of age, which might result into a decreased number of cases being reported (Cunningham *et al.*, 2015). Another reason behind the decline of the number of cervical lesions could be associated with high mortality rates of cervical cancer for women in their postmenopausal age.

4.2.3 Clinico-histopathological Classification of the Retrieved Cervical Biopsies

Among 149 women seeking care at KCMC from 1 May, 2017 to 10 May, 2018; 64 (43%) of cases were clinically presented with Per vaginal bleeding, followed by 43 (29%) cases with vaginal discharge and 35 (23%) cases of lower abdominal pain (Fig. 13).

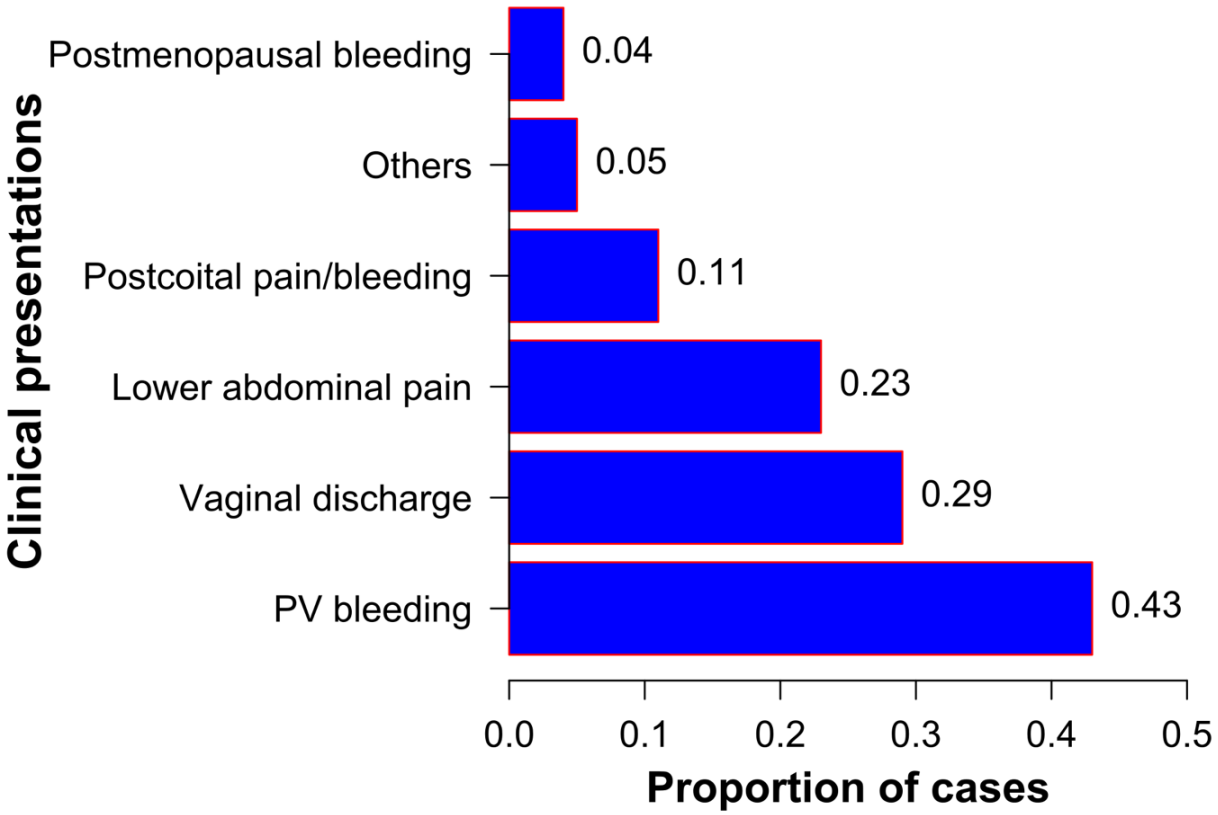

Figure 3: Distribution of clinical presentations among women seeking care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018

In contrast, a study conducted in India showed 43% for vaginal discharge, followed by 23% of vaginal bleeding as the most common symptoms (Dayal, 2018). These slightly similar findings can be associated with early invasive cervical cancer, which is generally asymptomatic (Eble *et al.*, 2003). In addition to this, clinical examination findings showed that 47.5% of all women seeking care at KCMC from 1 May, 2017 to 10 May, 2018 presented fungating mass that might indicate advanced stages of cervical lesions resulting in poor treatment responses.

In this study, squamous cell carcinoma accounted for 85 (85.9%) of total cervical cancer cases, followed by adenocarcinoma which accounted for 8 (8.1%), whilst others were undifferentiated carcinoma and neuroendocrine carcinoma. Both cervicitis and endocervical polyps were dominant inflammatory conditions, of which each accounted for 15 (45.5%) of all benign cervical lesions. On the other hand, mild dysplasia exhibited 7 (63.6%) of all pre-cancerous cervical lesions as seen in Table 4 and Fig. 4.

In contrast, the study conducted in Hawassa district, Southern Ethiopia showed that squamous cell carcinoma was the leading cervical lesion, with 83% of cervical cancer (Ameya & Yerakly, 2017). A potential thought for this similarity might be contributed by limited awareness and

screening programs in LMICs. Furthermore, this observed similarity might be caused by the fact that most of the cervical lesions have turned cancerous once they are diagnosed.

Table 4: Histopathological classification of cervical lesion among women seeking care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018

Histopathological classes	N (%)	% Total
Benign cervical lesion		
Cervicitis	15 (45.5)	10.1
Endocervical polyp/ leiomyomatous polyp	15 (45.5)	10.1
Cervical koilocytosis	1 (3.0)	0.7
Cervical papilloma	1 (3.0)	0.7
Nabothian cyst	1 (3.0)	0.7
Total	33 (100)	
Pre-cancerous cervical lesion		
CIN-1	7 (63.6)	4.7
CIN-3	3 (27.3)	2.0
CIN-2	1 (9.1)	0.7
Total	11 (100)	
Cancerous cervical lesion		
Squamous cell carcinoma	85 (85.9)	57.0
Adenocarcinoma	8 (8.1)	5.4
Undifferentiated carcinoma	5 (5.1)	3.4
Neuroendocrine carcinoma	1 (1.0)	0.7
Total	99 (100)	
Non-definitive diagnosis	6 (100)	4.0
Total	**149 (100)**	**100**

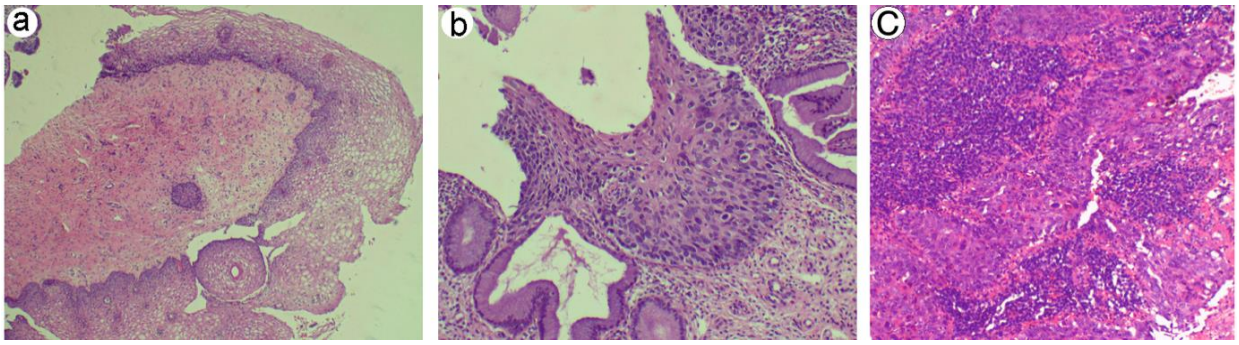

Figure 4: Selected haematoxylin & eosin-stained images of (a) normal tissue (b) carcinoma in situ (c) invasive squamous cell carcinoma of the cervix at 40x magnification of the retrieved cervical biopsies among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018

Moreover, the second predominant type of cervical disease in this study was a benign cervical lesion, of which 45.5% was cervicitis, which was almost similar to a study conducted in Southern Ethiopia that reported 53.6% of non-specific cervicitis among all non-cancerous lesions (Ameya & Yerakly, 2017).

In contrast, the study conducted in India reported 89.23% of chronic non-specific cervicitis (Jayakumar, 2015). Another study conducted in South-South, Nigeria reported 72.2% of chronic non-specific cervicitis among all inflammatory lesions (Nwachokor & Forae, 2013). Sexually transmitted infectious agents are the causative agents for cervicitis. It's reported that majorities of cervicitis are caused by *N. gonorrhoeae, C. trachomatis* and herpes simplex virus (Centres for Disease Control and Prevention [CDC], 2015).

However, histopathological differential diagnosis of cervicitis was not accomplished in our current study, which may be the reason for the above-observed differences in the prevalence of cervicitis in studies conducted in three countries. Polyps are most common in postmenopausal women, and when identified they are removed and histopathologically examined to prevent them from becoming cancerous (Gopalan *et al.*, 2017). However, the magnitude of endocervical polyp/ leiomyomatous polyp in our study was 45.5% for benign cervical lesion, and it was 10.1% of total cervical biopsies studied, which was lower than a study conducted in Nigeria (30.9%) (Faduyile *et al.*, 2017). Histopathological differential diagnosis of endocervical polyps was not performed.

4.3 Assessment of p16 and Topoisomerase II-alpha Biomarkers Expression

4.3.1 Clinico-histopathological Features and Biomarkers Expression

Out of 149, only 145 cervical biopsies for the immunohistochemistry staining of selected p16 and TOP2A biomarkers were included in this study. The included cervical biopsies were considered based on available clinical histopathological information and good morphology. Four (4) cervical biopsies were excluded due to missing tissue blocks, and lack of clinical histopathological information. Furthermore, immunohistochemistry staining results (Figure 5) revealed that most of the patients seeking cervical cancer care, 103 (71.0%) and 61 (42.1%), strongly expressed p16 (p16+) and TOP2A (TOP2A++) proteins, respectively (Table 2).

Importantly, the expressions of p16+ and TOP2A++ biomarkers were predominantly found among women aged 50–59 years, respectively (Figure 6). These study findings agree with previously reported findings that p16 and TOP2A immunoexpressions increased with severity of cervical lesions and may be used as a marker for classification of cervical lesions (Pandey *et al.*, 2018; Peres *et al.*, 2016; Shi *et al.*, 2019). Moreover, the relationships between p16 and TOP2A expression levels with histopathological classes for cervical cancer have been reported to be most associated with pre-cancerous and cancerous cervical lesions (Ding *et al.*, 2020; Pandey *et al.*, 2018; Peres *et al.*, 2016).

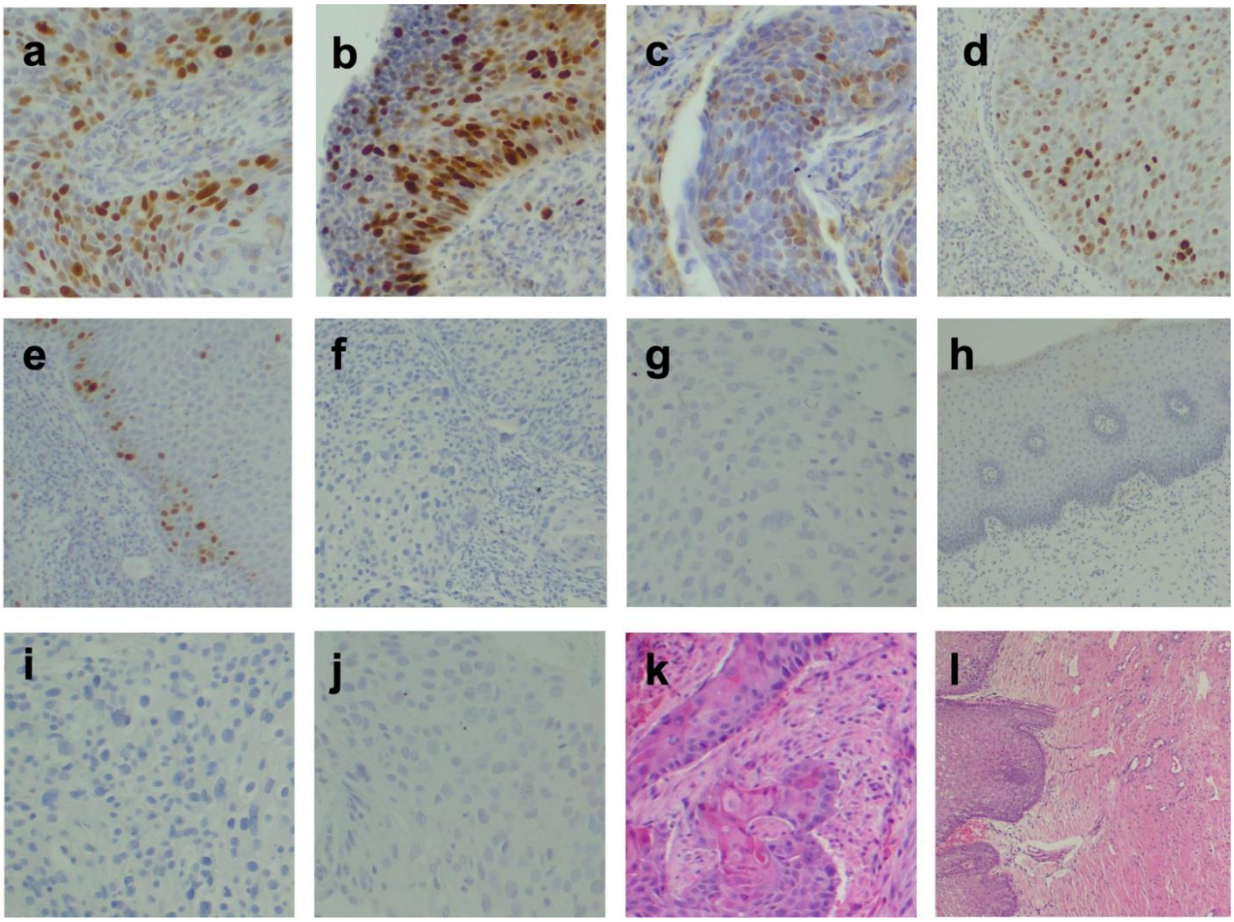

Figure 5: Monographs of the retrieved cervical biopsies among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018. Nucleus and cytoplasm were positively stained for (a-b) p16 at 200x for cancerous and precancerous cervical lesion respectively. Nucleus positively stained for (c-d) topoisomerase II-alpha at 200x for cancerous and precancerous cervical lesion respectively, and (e) topoisomerase II-alpha at 40x for the benign cervical lesion. Nucleus and cytoplasm negatively stained for (f-g) p16 at 100x and 200x for cancerous and precancerous cervical lesion respectively, and (h) p16 at 200x for the benign cervical lesion. Nucleus negatively stained for (i-j) topoisomerase II-alpha at 200x for cancerous and precancerous cervical lesion respectively; (k-l) hematoxylin and eosin staining at 100x and 40x for squamous cell carcinoma and normal cervix, respectively

4.3.2 Association of Clinico-histopathological Features and Biomarkers Expression

Age categories were not significantly associated with expression levels of p16 and TOP2A among women seeking cervical cancer care (Figure 6). In contrary, there was significant association between histopathological class with expression levels of p16 and TOP2A among women seeking cervical cancer care (p<0.001) (Table 5). However, no significant associations were observed between the histopathological subtypes of benign, precancerous, and cancerous cervical lesions, with p16 and TOP2A expressions in the studied population (Table 5). This implies that the expression of p16 and TOP2A biomarkers can be very useful in the prognosis of cervical cancer

across age groups. As both p16 and TOP2A play an important role in cellular proliferation, they may be accurately useful in defining the high-risk patients for cervical cancer, which need to be confirmed by large studies. Moreover, high expression of p16 and TOP2A was frequently observed in the age group of 40–49 years, which is considered the high-risk age group for pre-cancerous and cancerous cervical lesions. This can be explained by the limited cervical cancer screening practices and awareness campaigns among Tanzanian women in preventing the occurrence of the disease (Cunningham *et al.*, 2015; Lyimo & Beran, 2012; Moshi *et al.*, 2018; Perng *et al.*, 2013; Weng *et al.*, 2020). These challenges could be responsible for increased incidence and mortality rates of cervical cancer in older women (Grjibovski *et al.*, 2018; Weng *et al.*, 2020). Moreover, introduction of immunohistochemistry assessment of biomarkers may aid in the histopathological classification of cervical cancer and other cancers, and may assist in the early identification of Tanzanian women who are at high risk for recurrence of cervical cancer.

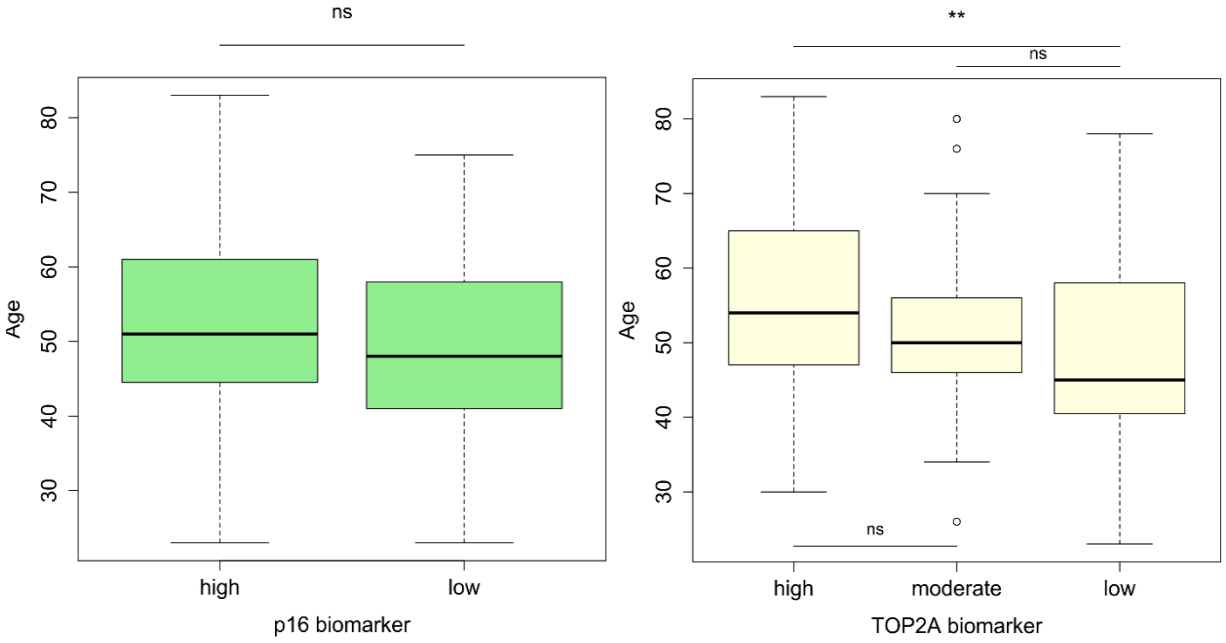

Figure 6: Distribution of p16 and topoisomerase II-alpha biomarkers expression across age-groups among women seeking care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018. The bold horizontal line within each column represents medians. Statistical significance is marked with double asterisks (Student's t-test, *p*=0.006) and ns stands for not significant

Table 5: Association of clinico-histopathological features and protein expressions among women seeking care at Kilimanjaro Christian Medical Centre from 1 May, 2017 to 10 May, 2018

SN	Variables	N=145 n (%)	p16 protein expression		*p*-value [a]	TOP2A protein expression			*p*-value
			Low n (%)	High n (%)		Low n (%)	Moderate n (%)	High n (%)	
1	Age (years)				0.813 [b]				0.256 [b]
	<30	5 (3.4)	2 (40.0)	3 (60.0)		3 (60.0)	1 (20.0)	1 (20.0)	
	30–39	21 (14.5)	7 (33.3)	14 (66.7)		11 (52.4)	3 (14.2)	7 (33.3)	
	40–49	48 (33.1)	16 (33.3)	32 (66.7)		20 (41.7)	12 (25.0)	16 (33.3)	
	50–59	34 (23.4)	8 (23.5)	26 (76.5)		11 (32.4)	7 (20.6)	16 (47.1)	
	60–69	25 (17.2)	7 (28.0)	18 (72.0)		7 (28.0)	2 (8.0)	16 (64.0)	
	>70	12 (8.3)	2 (16.7)	10 (83.3)		3 (25.0)	4 (33.3)	5 (41.7)	
2	Histopathological class				<0.001				<0.001
	Benign	33 (22.8)	33 (100)	0 (0)		30 (90.9)	2 (6.1)	1 (3.0)	
	Pre-cancerous	11 (7.6)	2 (18.2)	9 (81.8)		8 (72.7)	2 (18.2)	1 (9.1)	
	Cancerous	95 (65.5)	1 (1.1)	94 (98.9)		11 (11.6)	25 (26.3)	59 (62.1)	
	Non-definitive diagnosis	6 (4.1)	6 (100)	0 (0)		6 (100)	0 (0)	0 (0)	
3	Histopathological subtype								
	(a) Benign cervical lesion				1.000				0.479 [b]
	Cervicitis	15 (10.3)	15 (100)	0 (0)		13 (86.7)	1 (6.7)	1 (6.7)	
	Endocervical polyps	15 (10.3)	15 (100)	0 (0)		14 (93.3)	1 (6.7)	0 (0)	
	Others	3 (2.1)	3 (100)	0 (0)		2 (66.7)	1 (33.3)	0 (0)	
	(b) Pre-cancerous cervical lesion				1.000 [b]				0.241 [b]

SN	Variables	N=145 n (%)	p16 protein expression		*p*-value [a]	TOP2A protein expression			*p*-value
			Low n (%)	High n (%)		Low n (%)	Moderate n (%)	High n (%)	
	CIN-1	7 (4.8)	2 (28.6)	5 (71.4)		6 (85.7)	1 (14.3)	0 (0)	
	CIN-2	1 (0.7)	0 (0)	1 (100)		0 (0)	0 (0)	1 (100)	
	CIN-3	3 (2.1)	0 (0)	3 (100)		2 (66.7)	1 (33.3)	0 (0)	
(c)	Cancerous cervical lesion				0.122 [b]				0.584 [b]
	Squamous cell carcinoma	83 (57.2)	0 (0)	83 (100)		10 (12.0)	23 (27.7)	50 (60.2)	
	Adenocarcinoma	6 (4.1)	1 (16.7)	5 (83.3)		1 (16.7)	0 (0)	5 (83.3)	
	Others	6 (4.1)	0 (0)	6 (100)		0 (0)	2 (33.3)	4 (66.7)	
(d)	Non-definitive diagnosis	6 (4.1)	6 (100)	0 (0)		6 (100)	0 (0)	0 (0)	

[a] Fisher's exact test
[b] simulated *p*
CIN cervical intraepithelial lesion

4.3.3 Correlation between Histopathological Features and Biomarkers Expression

In the studied population, there was a highly significant positive correlation between p16 expression and cancerous cervical lesions (r=0.833, *p*=0.006). In contrary, a non-significant positive correlation between age and p16 expression was observed (r=0.110, *p*=0.643). By considering the effect size rather than the *p*-value due to the small sample size, there was a difference in the relationship between benign cervical lesions and p16 expression (r=-0.944, *p*<0.001) (Table 6). The overexpression levels of p16 could be due to pRb inactivation in the cancerous cervical lesions (Lambert *et al.*, 2006). In addition, a meta-analysis study reported the prognostic significance of p16 overexpression with improved overall survival and disease-free survival in cervical cancer patients (Lin *et al.*, 2014). However, stratification of the cancerous cervical lesions to assess p16 expression levels and HPV subtype was not performed in our study.

Nevertheless, there was no significant correlation between age and TOP2A expression in the studied population. A highly significant correlation between TOP2A expression and the cancerous cervical lesion was observed in the study population (r=0.687, *p*=0.005). The TOP2A overexpression was observed in 60.2% of squamous cell carcinoma and 83.3% of adenocarcinoma. The findings agree with those reported by Del Moral-Hernández *et al*. (2021), which showed that increased expression of TOP2A/MCM2 biomarkers to approximately 3-times increased the progression risk of HSIL to cervical cancer lesions. Similarly, TOP2A expression levels discriminated dysplastic and non-dysplastic FFPE cervical tissues with improved detection of CIN2+ (Depuydt *et al.*, 2011; Dixon *et al.*, 2017). Therefore, the cancerous cervical lesions strongly correlated with p16 and TOP2A expressions in the studied population. However, the expression of p16 also moderately correlated with TOP2A expression (Table 6) thereby, a 40% difference in the expression of p16 (high) versus TOP2A (high) biomarkers (100% versus 60%) was observed in the squamous cell carcinoma (Table 5) may imply that the p16 biomarker is more specific for squamous cell carcinoma than the TOP2A biomarker (Del Pino *et al.*, 2015; Pandey *et al.*, 2018; Peres *et al.*, 2016; Silva *et al.*, 2017). Furthermore, immunoscoring of the cut-off threshold for positive cells for both p16 and TOP2A biomarkers might vary between pathologists resulting in significant differences.

Additionally, a significant negative correlation between benign, and p16 and TOP2A expressions were observed in the studied population (r=-0.944 and -0.600, *p*<0.001 and 0.025,

respectively). In contrary, TOP2A expression and pre-cancerous cervical lesion in the studied population negatively correlated (Table 6). The findings disagree with those reported by Peres *et al*. (2021) that high TOP2A expression levels were observed in cervical smear samples than in cervical biopsies.

Table 6: Correlation matrix of p16 and topoisomerase II-alpha biomarkers expression with the histopathological factors among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre (n=139). The $p< 0.05$ was considered statistically significant

Parameters	Age	Benign	Precancerous	Cancerous	TOP2A	p16
Age	1.000					
Benign	-0.144	1.000				
Precancerous	-0.253	0.164	1.000			
Cancerous	0.278	-0.820*	-0.431	1.000		
TOP2A	0.240	-0.600*	-0.238	0.687**	1.000	
p16	0.110	-0.944***	0.052	0.833**	0.605*	1.000

* indicates significant ($p< 0.05$)

** indicates highly significant ($p < 0.01$)

*** indicates very highly significant ($p< 0.001$)

4.3.4 Diagnostic Performance and Strength of Associations between p16 and Topoisomerase II-alpha Biomarkers in Discriminating Cancerous and Non-cancerous Cervical lesions

The diagnostic utility of p16 and TOP2A immunohistochemistry was evaluated by classifying cancerous cervical lesions as positive cases, with benign and precancerous cervical lesions altogether classified as negative cases. The diagnostic performance of p16 was higher than that of TOP2A in the diagnosis of cancerous lesions from non-cancerous cervical lesions (sensitivity: 97.2% *versus* 77.6%, accuracy: 92.8% *versus* 87.8%). In contrary, a combination of p16 and TOP2A immunohistochemistry for the diagnostic utility was slightly lower than that of p16 or TOP2A alone in differentiating cancerous cervical lesions from non-cancerous cervical lesions (Table 7). These findings imply that p16 may be a potential biomarker in differentiating cancerous cervical lesions from benign and precancerous cervical lesions in the studied population.

Table 7: Diagnostic values of p16 and topoisomerase II-alpha immunohistochemistry in differentiating cancerous cervical lesions from benign and precancerous cervical lesions among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre (n = 139)

Methods	p16 % (95 % CI)	TOP2A[a] % (95% CI)	p16 *plus* TOP2A[a] % (95% CI)
Sensitivity	97.2 (85.5 – 99.9)	77.6 (63.4 – 88.2)	85.9 (76.6 – 92.5)
Specificity	91.3 (84.1 – 95.9)	93.3 (86.1 – 97.5)	92.2 (87.5 – 95.6)
Accuracy	92.8 (87.2 – 96.5)	87.8 (81.1 – 92.7)	90.3 (86.2 – 93.5)

CI confidence intervals

[a] modified expression levels (low: moderate + high)

Nevertheless, cancerous cervical lesions were statistically correlated with p16 expression (p<0.001). Likewise, cancerous cervical lesions were significantly correlated with expression of TOP2A (p=0.014). In contrary, the combined expression of p16 and TOP2A biomarkers was non-significantly correlated with cancerous cervical lesions in the studied population (Table 8). Moreover, the age-adjusted odds ratio for the cancerous cervical lesions (relative to the benign and precancerous cervical lesions) were 1.142 (95% CI: 1.059–1.232) and 1.046 (95% CI: 1.008–1.085) for the expression levels of p16 and TOP2A biomarkers, respectively. In addition, the age-adjusted odds ratio for the cancerous cervical lesions (relative to the benign and precancerous cervical lesions) was 0.989 (95% CI: 0.946–1.034) for the combination of p16 and TOP2A biomarkers (Table 8). These findings corroborate with previously reported studies that used cervical scraps/ FFPE tissues, which showed that progression of LSIL/CIN-1 to cervical cancer lesions was conferred by p16, TOP2A, p16 and TOP2A [ProEx C] (Del Moral-Hernández *et al.*, 2021; Ozaki *et al.*, 2011; Zhang & Shen, 2018). Overall, the findings support the associations of p16 and TOP2A biomarkers in the development of cervical cancer lesions.

Table 8: Multivariate regression analysis and the strength of association between expression levels of p16 and topoisomerase II-alpha in differentiating cancerous cervical lesions from benign and precancerous cervical lesions among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre (n = 139)

Variable [a]	Factor	Overall model significance $p<0.001$			
		AOR	95% CI for AOR		*p*-value
p16	Low	1			
	High	1.142	1.059	1.232	<0.001
TOP2A	Low	1			
	High	1.046	1.008	1.085	0.015
p16 *and* TOP2A	Low	1			
	High	0.989	0.946	1.034	0.638

CI confidence intervals

AOR age-adjusted odds ratio

[a] benign and precancerous cervical lesions were altogether used as a reference group

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Findings from the study suggest that over-expression of p16 and TOP2A proteins significantly correlated with cancerous cervical lesions, and may be promising biomarkers for differentiating cancerous cervical lesions from non-cancerous cervical lesions in the studied population. However, further investigation and feasibility studies are still needed on these biomarkers before the early diagnosis of cervical cancer especially in Tanzania regions that have a high-burden of cervical cancer cases.

5.2 Recommendations

The following are the recommendations derived from the above conclusion:

(i) There is an adequate need to promptly investigate other cell proliferation biomarkers such as p16, Ki-67, MYC, cyclins, telomerase, and replication complex proteins for cervical carcinogenesis in Tanzania, to improve early detection and diagnosis of cervical cancer lesions.

(ii) Importantly, a large study involving a combination of p16 and TOP2A proteins and their messenger ribonucleic acids (mRNAs), and their association with hrHPV subtypes and differential histopathological diagnosis can provide a more detailed pattern in the study area.

(iii) Progressive community-based awareness campaigns and screening programs for cervical cancer lesions are highly needed in the study area and country at large, to reduce many reported cases of advanced cervical cancerous lesions.

(iv) In addition, the diagnostic performances of p16 and TOP2A biomarkers need to be validated in a clinical setting and compared to readily available commercial diagnostic assays for cervical cancer.

(v) A low-cost, simple and rapid HPV tests are highly needed in our clinical setting considering the rampant increase of cancer-related morbidity and mortality rates in the

studied area and Tanzania at large rather than immunohistological tests, which are expensive.

REFERENCES

Ameya, G., & Yerakly, F. (2017). Characteristics of cervical disease among symptomatic women with histopathological sample at Hawassa University referral hospital, Southern Ethiopia. *BMC Women's Health, 17*(1), 91-97.

Ardahan, M., & Temel, A. B. (2011). Visual inspection with acetic acid in cervical cancer screening. *Cancer Nursing, 34*(2), 158-163.

Beddoe, A. M. (2019). Elimination of cervical cancer: Challenges for developing countries. *Ecancer, 13*, 975-980.

Black, E., & Richmond, R. (2018). Prevention of cervical cancer in sub-Saharan Africa: The advantages and challenges of HPV vaccination. *Vaccines, 6*(3), 61-68.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394-424.

Brown, C. A., Bogers, J., Sahebali, S., Depuydt, C. E., De Prins, F., & Malinowski, D. P. (2012). Role of protein biomarkers in the detection of high-grade disease in cervical cancer screening programs. *Journal of Oncology, 2012*, 1-11.

Bruni, L., Albero, G., Serrano, B., Mena, M., Gómez, D., Muñoz, J., Bosch, F., & de Sanjosé, S. (2019). *ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human papillomavirus and related diseases in Tanzania*. www.google.com

Centres for Disease Control and Prevention. (2015). *Sexually Transmitted Diseases Treatment Guidelines.* https://www.cdc.gov/std/tg2015/urethritis-and-cervicitis.htm

Cohen, P. A., Jhingran, A., Oaknin, A., & Denny, L. (2019). Cervical cancer. *The Lancet, 393*(10167), 169-182.

Crow, J. M. (2012). HPV: The global burden. *Nature, 488*(7413), 2-3.

Cunningham, M. S., Skrastins, E., Fitzpatrick, R., Jindal, P., Oneko, O., Yeates, K., Booth, C. M., Carpenter, J., & Aronson, K. J. (2015). Cervical cancer screening and HPV vaccine acceptability among rural and urban women in Kilimanjaro Region, Tanzania. *Biomedical Journal Open*, *5*(3), 1-9.

Dartell, M. A., Rasch, V., Iftner, T., Kahesa, C., Mwaiselage, J. D., Junge, J., Gernow, A., Ejlersen, S. F., Munk, C., & Kjaer, S. K. (2014). Performance of visual inspection with acetic acid and human papillomavirus testing for detection of high-grade cervical lesions in HIV positive and HIV negative Tanzanian women. *International Journal of Cancer*, *135*(4), 896-904.

Dasari, S., Wudayagiri, R., & Valluru, L. (2015). Cervical cancer: Biomarkers for diagnosis and treatment. *Clinica Chimica Acta*, *445*, 7-11.

Dayal, S. (2018). Clinico-histological analysis of non-neoplastic lesions of cervix. *Journal of Pathology of Nepal*, *8*(1), 1276-1279.

De Matos, L. L., Trufelli, D. C., De Matos, M. G. L., & da Silva Pinhal, M. A. (2010). Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker Insights*, *5*, 9-20.

Del Moral-Hernández, O., Hernández-Sotelo, D., del Carmen Alarcón-Romero, L., Mendoza-Catalán, M. A., Flores-Alfaro, E., Castro-Coronel, Y., Ortiz-Ortiz, J., Leyva-Vázquez, M. A., Ortuño-Pineda, C., & Castro-Mora, W. (2021). TOP2A/MCM2, p16INK4a, and cyclin E1 expression in liquid-based cytology: A biomarkers panel for progression risk of cervical premalignant lesions. *Cancer*, *21*(1), 1-13.

Del Pino, M., Svanholm-Barrie, C., Torné, A., Marimon, L., Gaber, J., Sagasta, A., Persing, D. H., & Ordi, J. (2015). mRNA biomarker detection in liquid-based cytology: A new approach in the prevention of cervical cancer. *Modern Pathology*, *28*(2), 1-9.

Denny, L. (2015). *Cervical cancer screening tests: Visual inspection methods*. https:// www. uptodate. com/ contents/ cervical- cancer- screening- tests-visual-inspection -methods/ print #:~: text = Visual % 20 inspection %20 can %20 be %20 performed, with % 20 Lugol's% 20Iodine%20(VILI Accessed 02 March 2021

Depuydt, C. E., Makar, A. P., Ruymbeke, M. J., Benoy, I. H., Vereecken, A. J., Bogers, J. J. J. C. E., & Biomarkers, P. (2011). BD-ProExC as adjunct molecular marker for improved detection of CIN2+ after HPV primary screening. *Cancer Epidemiology, Biomarkers and Prevention, 20*(4), 628-637.

Dias, T. C., Longatto-Filho, A., & Campanella, N. C. (2020). Human papillomavirus genotyping as a tool for cervical cancer prevention: From commercially available human papillomavirus DNA test to next-generation sequencing. *Future Science OA, 6*(9), 1-8.

Ding, L., Song, L., Zhao, W., Li, X., Gao, W., Qi, Z., & Wang, J. (2020). Predictive value of p16INK4a, Ki-67 and ProExC immuno-qualitative features in LSIL progression into HSIL. *Experimental and Therapeutic Medicine, 19*(4), 2457-2466.

Dixon, E. P., King, L. M., Nelson, R., Simkins, S. G., Knapp, S. L., Brough, G. H., Lenz, K. L., Henderson, D. T., Whitehead, C. M., & Hessling, J. (2017). Characterization and clinical validation of MCM2 and TOP2A monoclonal antibodies in the BD ProEx™ C assay: An immunoassay which detects aberrant S-phase induction in cervical tissue. *Journal of Immunological Methods, 442*, 35-41.

Duraiyan, J., Govindarajan, R., Kaliyappan, K., & Palanisamy, M. (2012). Applications of immunohistochemistry. *Journal of Pharmacy and Bioallied Sciences, 4*(2), 307-309.

Eble, J. N., Tavassoli, F. A., & Devilee, P. (2003). *Pathology and genetics of tumours of the breast and female genital organs*. Lyon: IARC Press.

Ergete, W., & Tesfaye, A. (2001). Histopathological findings of post-menopausal bleeding in Ethiopian women. *Ethiopian Journal of Health Development, 15*(1), 39-44.

Faduyile, F. A., Soyemi, S. S., Wright, K. O., & Osuolale, F. (2017). Histopathological study of surgical cervical biopsies in Lagos, Nigeria. *Tropical Journal of Obstetrics and Gynaecology, 34*(2), 124-128.

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D., Piñeros, M., Znaor, A., & Bray, F. (2019). Estimating the global cancer incidence and mortality in 2018:

GLOBOCAN sources and methods. *International Journal of Cancer, 144*(8), 1941-1953.

Gopalan, U., Rajendiran, S., & Karnaboopathy, R. (2017). Clinicopathological analysis of cervical polyps. *International Journal of Reproduction, Contraception, Obstetrics and Gynecology, 6*, 1526-1529.

Grjibovski, A. M., Dubovichenko, D., Saduakassova, S., Zhatkanbayeva, G., Omarova, G., Shalgumbayeva, G., Valkova, L., & Valkov, M. Y. (2018). Incidence, mortality and determinants of survival from cervical cancer in Northwest Russia: A registry-based cohort study. *International Health, 10*(2), 92-99.

Gustinucci, D., Passamonti, B., Cesarini, E., Butera, D., Palmieri, E. A., Bulletti, S., Carlani, A., Staiano, M., D'Amico, M. R., & D'Angelo, V. (2012). Role of p16INK4a cytology testing as an adjunct to enhance the diagnostic specificity and accuracy in human papillomavirus-positive women within an organized cervical cancer screening program. *Acta Cytologica, 56*(5), 506-514.

Güzel, C., van Sten-van't Hoff, J., de Kok, I. M., Govorukhina, N. I., Boychenko, A., Luider, T. M., & Bischoff, R. (2021). Molecular markers for cervical cancer screening. *Expert Review of Proteomics*, 1-17.

Hwang, S. J., & Shroyer, K. R. (2012). Biomarkers of cervical dysplasia and carcinoma. *Journal of Oncology, 2012*, 1-9.

Ibeanu, O. A. (2011). Molecular pathogenesis of cervical cancer. *Cancer Biology and Therapy, 11*(3), 295-306.

International Agency for Research on cancer. (2021). GLOBOCAN 2020: GLOBOCAN Estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Retrieved from https://gco.iarc.fr/today/data/factsheets/cancers/23-Cervix-uteri-fact-sheet.pdf Accessed 10 March 2021

Jayakumar, N. K. B. (2015). Cervicitis: How often is it non-specific! *Journal of Clinical Diagnostic Research, 9*(3), 11-12.

Katanga, J., Kjaer, S. K., Manongi, R., Wu, C. S., Iftner, T., Waldstrom, M., Pembe, A. B., Mwaiselage, J., & Rasch, V. (2019). Performance of care HPV, hybrid capture 2 and visual inspection with acetic acid for detection of high-grade cervical lesion in Tanzania: A cross-sectional study. *PLoS One, 14*(6), 1-13.

Kurman, R. J., Carcangiu, M. L., Herrington, C. S., & Young, R. H. (2014). *WHO classification of tumours of female reproductive organs*. Lyon: IARC Press.

Lambert, A. P. F., Anschau, F., & Schmitt, V. M. (2006). p16INK4A expression in cervical premalignant and malignant lesions. *Experimental and Molecular Pathology, 80*(2), 192-196.

Li, Y., Liu, J., Gong, L., Sun, X., & Long, W. (2019). Combining HPV DNA load with p16/Ki-67 staining to detect cervical precancerous lesions and predict the progression of CIN1–2 lesions. *Virology Journal, 16*(1), 1-9.

Lin, J., Albers, A. E., Qin, J., & Kaufmann, A. M. (2014). Prognostic significance of overexpressed p16 INK4a in patients with cervical cancer: A meta-analysis. *PLoS One, 9*(9), 1-7.

Lyimo, F. S., & Beran, T. N. (2012). Demographic, knowledge, attitudinal, and accessibility factors associated with uptake of cervical cancer screening among women in a rural district of Tanzania: Three public policy implications. *BMC Public Health, 12*(1), 22-29.

Marcus, A., Wang, Z., & Mittal, K. (2017). Utility of p16 and Ki-67 expression in cervical biopsy with cervical intraepithelial neoplasia 2 to predict diagnosis in subsequent cervical excision specimen. *Journal of Cervical Cancer Research, 1*(1), 6-9.

McGraw, S. L., & Ferrante, J. M. (2014). Update on prevention and screening of cervical cancer. *World Journal of Clinical Oncology, 5*(4), 744-752.

Mishra, A., & Verma, M. (2010). Cancer biomarkers: Are we ready for the prime time? *Cancers, 2*(1), 190-208.

Moshi, F. V., Vandervort, E. B., & Kibusi, S. M. (2018). Cervical cancer awareness among women in Tanzania: An analysis of data from the 2011-12 Tanzania HIV and Malaria indicators survey. *International Journal of Chronic Diseases, 2018*, 1-7.

Ngoma, T., Muwonge, R., Mwaiselage, J., Kawegere, J., Bukori, P., & Sankaranarayanan, R. (2010). Evaluation of cervical visual inspection screening in Dar es Salaam, Tanzania. *International Journal of Gynecology and Obstetrics, 109*(2), 100-104.

Nwachokor, F., & Forae, G. (2013). Morphological spectrum of non-neoplastic lesions of the uterine cervix in Warri, South-South, Nigeria. *Nigerian Journal of Clinical Practice, 16*(4), 429-432.

Orang'o, E. O., Were, E., Rode, O., Muthoka, K., Byczkowski, M., Sartor, H., Broeck, D. V., Schmidt, D., Reuschenbach, M., von Knebel Doeberitz, M., & Bussmann, H. (2020). Novel concepts in cervical cancer screening: A comparison of VIA, HPV DNA test and p16 INK4a/Ki-67 dual stain cytology in Western Kenya. *Infectious Agents and Cancer, 15*(1), 1-10.

Ozaki, S., Zen, Y., & Inoue, M. (2011). Biomarker expression in cervical intraepithelial neoplasia: Potential progression predictive factors for low-grade lesions. *Human Pathology, 42*(7), 1007-1012.

Pandey, A., Chandra, S., Nautiyal, R., & Shrivastav, V. (2018). Expression of p16INK4a and human papillomavirus 16 with associated risk factors in cervical premalignant and malignant lesions. *South Asian Journal of Cancer, 7*(4), 236-239.

Peres, A. L., e Silva, K. M. P., de Araújo, R. F. F., de Lima Filho, J. L., de Melo Júnior, M. R., Martins, D. B. G., & de Pontes Filho, N. T. (2016). Immunocytochemical study of TOP2A and Ki-67 in cervical smears from women under routine gynecological care. *Journal of Biomedical Science, 23*(1), 42-49.

Perng, P., Perng, W., Ngoma, T., Kahesa, C., Mwaiselage, J., Merajver, S. D., & Soliman, A. S. (2013). Promoters of and barriers to cervical cancer screening in a rural setting in Tanzania. *International Journal of Gynecology and Obstetrics, 123*(3), 221-225.

Qureshi, S., Das, V., & Zahra, F. (2010). Evaluation of visual inspection with acetic acid and Lugol's iodine as cervical cancer screening tools in a low-resource setting. *Tropical Doctor, 40*(1), 9-12.

Rusling, J. F., Kumar, C. V., Gutkind, J. S., & Patel, V. (2010). Measurement of biomarker proteins for point-of-care early detection and monitoring of cancer. *Analyst, 135*(10), 2496-2511.

Sachan, P. L., Singh, M., Patel, M. L., & Sachan, R. (2018). A study on cervical cancer screening using pap smear test and clinical correlation. *Asia-Pacific Journal of Oncology Nursing, 5*(3), 337-341.

Sahasrabuddhe, V. V., Luhn, P., & Wentzensen, N. (2011). Human papillomavirus and cervical cancer: Biomarkers for improved prevention efforts. *Future Microbiology, 6*(9), 1083-1098.

Sankaranarayanan, R., Nessa, A., Esmy, P. O., & Dangou, J. M. (2012). Visual inspection methods for cervical cancer prevention. *Best Practice & Research Clinical Obstetrics and Gynaecology, 26*(2), 221-232.

Schiffman, M., Doorbar, J., Wentzensen, N., De Sanjosé, S., Fakhry, C., Monk, B. J., Stanley, M. A., & Franceschi, S. (2016). Carcinogenic human papillomavirus infection. *Nature Reviews Disease Primers, 2*(1), 1-20.

Sherwani, R., Khan, T., Akhtar, K., Zeba, A., Siddiqui, F., Rahman, K., & Afsan, N. (2007). Conventional Pap smear and liquid based cytology for cervical cancer screening-A comparative study. *Journal of Cytology, 24*(4), 167-172.

Shi, Q., Xu, L., Yang, R., Meng, Y., & Qiu, L. (2019). Ki-67 and p16 proteins in cervical cancer and precancerous lesions of young women and the diagnostic value for cervical cancer and precancerous lesions. *Oncology Letters, 18*(2), 1351-1355.

Sigurdsson, K. (2013). Is a liquid-based cytology more sensitive than a conventional Pap smear? *Cytopathology, 24*(4), 254-263.

Silva, D. C., Goncalves, A. K., Cobucci, R. N., Mendonca, R. C., Lima, P. H., & Júnior, G. C. (2017). Immunohistochemical expression of p16, Ki-67 and p53 in cervical lesions–A systematic review. *Pathology - Research and Practice, 213*(7), 723-729.

Small, W., Bacon, M. A., Bajaj, A., Chuang, L. T., Fisher, B. J., Harkenrider, M. M., Jhingran, A., Kitchener, H. C., Mileshkin, L. R., & Viswanathan, A. N. (2017). Cervical cancer: A global health crisis. *Cancer, 123*(13), 2404-2412.

Sun, H., Shen, K., & Cao, D. (2019). Progress in immunocytochemical staining for cervical cancer screening. *Cancer Management and Research, 11*, 1817-1827.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 0*(0), 1-41.

Tornesello, M. L., Buonaguro, L., Giorgi-Rossi, P., & Buonaguro, F. M. (2013). Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. *BioMed Research International, 2013*, 1-10.

Volgareva, G., Zavalishina, L., Andreeva, Y., Frank, G., Krutikova, E., Golovina, D., Bliev, A., Spitkovsky, D., Ermilova, V., & Kisseljov, F. (2004). Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. *Cancer, 4*(1), 1-10.

Weng, Q., Jiang, J., Haji, F. M., Nondo, L. H., & Zhou, H. (2020). Women's knowledge of and attitudes toward cervical cancer and cervical cancer screening in Zanzibar, Tanzania: A cross-sectional study. *Cancer, 20*(1), 63-74.

Wu, M. Z., Wang, S., Zheng, M., Tian, L. X., Wu, X., Guo, K. J., Zhang, Y., & Wu, G. P. (2019). The diagnostic utility of p16 immunostaining in differentiating cancer and HSIL from LSIL and benign in cervical cells. *Cell Transplantation, 28*(2), 195-200.

Zhang, X., & Shen, D. (2018). p16INK4a and Ki-67 measurement predict progression of cervical low-grade squamous intraepithelial lesion. *International Journal of Clinical and Experimental Pathology, 11*(8), 4109-4116.

APPENDICES

Appendix 1: Ethical clearance

THE UNITED REPUBLIC OF TANZANIA

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NIMR/HQ/R.8a/Vol. IX/2764

09th May 2018

Zavuga Zuberi
C/o Dr. Elingarami Nkya
Nelson Mandela African Institute of Science and Technology
P.O. Box 447
Arusha

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Expression of protein biomarkers in females with pre-malignant cervical lesions in Northern Tanzania (Zuberi Z. *et al.*) whose supervisor is Dr. Elingarami Nkya of Nelson Mandela African Institute of Science and Technology has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Site: Kilimanjaro.

Approval is valid for one year: 09th May 2018 to 08th May 2019.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: RMO of Kilimanjaro.
DMO/DED of Selected districts.

Appendix 2: Retrieval form for the archived cervical biopsies, slides and patient's data

Study area: Kilimanjaro Christian Medical Centre, Tanzania

Patient's hospital registration No.: _____

Laboratory ID: _____

Patient's age: _____ years

Date of examination: _____

<table>
<tr><td colspan="8">Retrieved biosamples (put a ✓ in applicable boxes)</td></tr>
<tr><td colspan="3">1. Tissue block ☐</td><td colspan="2">2. Stained H&E slide ☐</td><td colspan="3">3. Blocken/missing H&E slide ☐</td></tr>
<tr><td colspan="8">Clinical presentations of the patient (select one box from each of numbers from 1-5)</td></tr>
<tr><td colspan="2">1. Vaginal discharge
Yes ☐
No ☐</td><td colspan="2">2. Vaginal bleeding
Yes ☐
No ☐</td><td colspan="2">3. Abdominal pain
Yes ☐
No ☐</td><td colspan="2">4. Postcoital pain or bleeding
Yes ☐ No ☐</td></tr>
<tr><td colspan="8">5. Postmenopausal bleeding
Yes ☐
No ☐</td></tr>
<tr><td colspan="8">Histology findings (put a ✓ in ONLY one of the following boxes)</td></tr>
<tr><td colspan="2">Benign lesions</td><td colspan="2">Pre-cancerous lesions</td><td colspan="2">Cancerous lesions</td><td colspan="2">Non-definitive diagnosis</td></tr>
<tr><td>Cervicitis</td><td>☐</td><td>CIN-1</td><td>☐</td><td>Adenocarcinoma</td><td>☐</td><td colspan="2">Yes ☐</td></tr>
<tr><td>Cervical polyp</td><td>☐</td><td>CIN-2</td><td>☐</td><td rowspan="5">Squamous cell carcinoma</td><td rowspan="5">☐</td><td colspan="2" rowspan="5">No ☐</td></tr>
<tr><td>Leiomyoma polyp</td><td>☐</td><td>CIN-3</td><td>☐</td></tr>
<tr><td>Cervical koilocytosis</td><td>☐</td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>Cervical papilloma</td><td>☐</td></tr>
<tr><td>Nabothian cyst</td><td>☐</td></tr>
<tr><td colspan="8">Supplementary information</td></tr>
</table>

RESEARCH OUTPUTS

Output 1: Journal paper

Zuberi, Z., Mremi, A., Chilongola, J. O., Semango, G., & Sauli, E. (2021). Expression of p16 and TOP2A biomarkers associated with clinico-histopathological features among women seeking cervical cancer care in Tanzania. *PLoS One*, 16 (10): 1-15.

Output 2: Poster presentation

Zuberi, Z., Chilongola, J. O., & Sauli, E. (2021). *Diagnostic performance of p16 and TOP2A biomarkers in differentiating cancerous and non-cancerous cervical lesions*.

Diagnostic performance of p16 and TOP2A biomarkers in differentiating cancerous and non-cancerous cervical lesions

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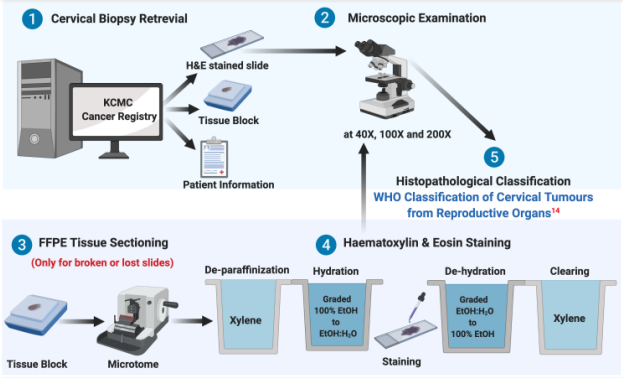
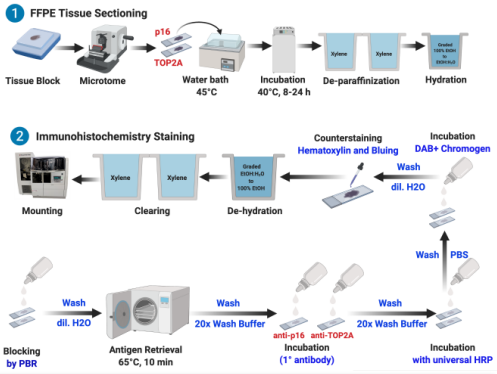
Background

- Cervical cancer (CC) is the fourth frequently diagnosed cancer that affects women ~ 265,000 deaths in LMICs globally.[1] It is the leading cause of female cancers, with the crude incidence rate of 32.7% and; crude mortality rate of 22.4% in 2018 in Tanzania.[2]
- About 80% reduction of CC mortality and incidence rates have been reported in HICs. However, early diagnosis programs have been unable to control CC in LMICs like Tanzania, because of lack, or limited access and low performances of the available methods.[3]
- Several cancer biomarkers when individually applied, may not detect CC as it develops, making them unsuitable for screening and diagnosis of CC.[4] Expression levels of p16 and TOP2A biomarkers have been reported in CC diagnosis in HICs[5], which necessitates the need for their feasibility study in the setting of LMICs like Tanzania. Therefore, this study was conceived to evaluate the utility of p16 and TOP2A as potential biomarkers in dysplastic and malignant alteration of cervical epithelium among women seeking cervical cancer care at KCMC, Tanzania.

Methods





Results

Methods	p16 % (95 % CI)	TOP2A[a] % (95% CI)	p16 plus TOP2A[a] % (95% CI)
Sensitivity	97.2 (85.5 – 99.9)	77.6 (63.4 – 88.2)	85.9 (76.6 – 92.5)
Specificity	91.3 (84.1 – 95.9)	93.3 (86.1 – 97.5)	92.2 (87.5 – 95.6)
Accuracy	92.8 (87.2 – 96.5)	87.8 (81.1 – 92.7)	90.3 (86.2 – 93.5)

CI confidence intervals
[a] modified expression levels (low: moderate + high)

Table 1. Diagnostic values of p16 and TOP2A immunohistochemistry in differentiating cancerous cervical lesions from benign and precancerous cervical lesions among women seeking cervical cancer care at Kilimanjaro Christian Medical Centre (n = 139)

Conclusion and Future prospects

- Over-expression of p16 and TOP2A proteins significantly correlated with cancerous cervical lesions, and may be promising biomarkers for differentiating cancerous cervical lesions from non-cancerous cervical lesions.
- However, further investigation and feasibility studies are still needed on these biomarkers before the early diagnosis of cervical cancer especially in Tanzania.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018; 68(6):394–424.
2. Bruni L, Albero G, Serrano B, Mena M, Go´mez D, Muñoz J, et al. Human papillomavirus and related diseases in Tanzania. Summary Report 17 June 2019. [Internet].
3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015; 136(5): E359–E86.
4. Sun H, Shen K, Cao D. Progress in immunocytochemical staining for cervical cancer screening. *Cancer Manag Res.* 2019; 11:1817.
5. Ding L, Song L, Zhao W, Li X, Gao W, Qi Z, et al. Predictive value of p16INK4a, Ki-67 and ProExC immuno-qualitative features in LSIL progression into HSIL. *Exp Ther Med.* 2020; 19(4):2457–66.
14. Kurman RJ, Carcangiu ML, Herrington CS, Young RH. WHO classification of tumours of female reproductive organs. Lyon: International Agency for Research on Cancer; 2014.