Role of Dual-biomarkers p-16 and Ki-67 Immunocytology in Human Papillomavirus (HPV) DNA Testing and HPV 16/18 Genotyping based Cervical Cancer Screening in Myanmar

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Simultaneous detection of dual-biomarker p-16 and Ki-67 immunocytology provides a strong indicator of the presence of transforming Human Papillomavirus (HPV) infections. Co-expression of p16 and Ki-67 in the same cell is strongly associated with established high-grade cervical diseases. The objectives were to conduct Human Papillomavirus (HPV) DNA testing and HPV 16/18 Genotyping based Cervical Cancer Screening among married women in Bogalay Township, Ayeyarwady Region, Myanmar and to apply Liquid Based Cytology (LBC) and dual-biomarker p-16/Ki-67 immunocytology among HPV positive women. It was a prospective cross-sectional descriptive study. A total of 677 married women (mean age-40-years; range 30-55) residing in Bogalay Township, Ayeyarwady Region, Myanmar were screened in 2020. HPV-DNA testing and HPV-16/18 genotyping was performed in Department of Medical Research using Cobas 4800 system. It is an automated real-time-polymerase-chain-reaction and nucleic-acid-hybridization test for detection of 14-HR-HPV genotypes. Positive HPV cases were followed by liquid-based-cytology (LBC) and dual-biomarker p-16/Ki-67 immunocytology. Histopathology was performed in HPV-positive high-grade cervical lesions. HPV was identified in 4.6% (31/677) of screened women in Bogalay Township, Ayeyarwady Region, Myanmar. Among HPV positive cases, HPV-16 was 22.6% (7/31), HPV-18 was 6.5% (2/31) and pooled-12-other-HR-HPV-genotypes (HPV-31,-33,-35,-39,-51,-52,-56,-58,-59,-66 and-68) were 70.9% (22/31). In liquid-based cytology, 87.1% (27/31) of HPV positive women had cervical cytological abnormalities comprising; 16.1% (5/31) had high grade squamous intraepithelial lesion (HSIL), 48.4% (15/31) had low grade squamous intraepithelial lesion (LSIL) and 22.6% (7/31) had atypical squamous cells of undetermined significance (ASCUS). Only 12.9% (4/31) were negative for intraepithelial lesion or malignancy (NILM). In immunocytology of HPV-positive women, all HSIL (100%) (5/5) cases, 66.7% (10/15) of LSIL, 14.3% (1/7) of ASCUS was positive with both p-16 and Ki-67 immuno-biomarker but negative in NILM cases. In histopathology of HPV-positive/HSIL women, 40% had cervical intraepithelial neoplasia (CINII), 40% (CINIII) and 20% (invasive squamous cell cancer of cervix). This study highlighted that dual-biomarkers p-16 and Ki-67 immunocytology was very useful to confirm the high-grade cervical lesions of HPV positive women. In addition, triage with this dual-biomarker immunocytology among low-grade cervical lesions of HPV positive women reduce the number of women that require referral to colposcopy. Results in more (chance) disease detection (rate) without increasing the colposcopy rate can aid clinicians making decisions.

Keywords: HPV DNA testing, p-16/Ki-67 immunocytology, Cervical Cancer Screening

INTRODUCTION

Worldwide, cervical cancer is the fourth most common cancer among women worldwide, with an estimated 604,127 new cases and 341,831 deaths in 2020 (GLOBOCAN 2020). In Southeast Asia (SEA), incidence and mortality of cervical cancer are about 68,623 cases and 38,530 deaths respectively.[1] In Myanmar, cervical cancer is the first most

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common female cancer and the first leading cause of cancer deaths in women aged 15 to 44 years (IARC 2021). Age standardized incidence rate of cervical cancer were 22.6% in Myanmar, 17.8% in Southeast Asia and 13.3% in the world. Age standardized mortality rate of cervical cancer were 14.4% in Myanmar, 9.9% in South East Asia and 7.3% in the world.[2]

Human Papillomavirus (HPV) infection is among the most common sexually transmitted infections. Persistent infection with high-risk types is associated with precancerous and cancerous lesions.[3] HPV types 16 and 18 are the most common high-risk types worldwide and are considered to be responsible for more than 70% of all cervical cancer cases.[4, 5] The high sensitivity of human papillomavirus (HPV) testing and its reassurance of a low risk of cervical cancer in HPV-negative women has fostered a shift from cytology-based screening toward HPV testing as the primary cervical cancer screening strategy.[6, 7]

Multiple professional societies, such as the American Cancer Society (ACS),[8] the American Society for Colposcopy and Cervical Pathology (ASCCP),[9] the European Society of Gynecologic Oncology and the European Federation of Colposcopy (ESGO-EFC),[10] and the US Preventive Services Task Force (USPSTF),[11] have recommended primary HPV testing to be preferred for cervical cancer screening. In Myanmar, national cervical cancer screening guideline was established from Maternal and Reproductive Health Division, Department of Public Health, Ministry of Health in August 2018. In this guideline, primary HPV testing will be employed in both rural and urban areas when the resources are sufficient.[12]

Managing human papillomavirus (HPV)-positive women in cervical cancer screening is very important. Many HPV infections are transient and can be eliminated by the body's immune system. As such, it is neither feasible nor efficient for all HPV-positive women to have a referral for colposcopy. HPV-positive women should be further triaged by another test to avoid unnecessary colposcopy referral. Cytology is a preferred examination for triaging HPV positive women because of its high specificity, but it is a subjective judgment, and the accuracy depends on the professional level of the cytologist.[13] Therefore, additional triage strategies are needed to distinguish those HPV-positive women who are at a high risk and need colposcopy from those who can safely return to routine screening. According to the current triage strategies for primary cervical screening, women with HPV16/18-positive results are referred to colposcopy and women positive for 12 other HPV genotyping are further triaged by Pap cytology.[6] However, the reliance on morphologic assessment and relative subjectivity, for which high expertise is required, limits the overall effectiveness of Pap cytology as the optimal "second line" triage test. Thus, there is a need to investigate new triage strategies for primary HPV screening.

The p16/Ki-67 dual-stained cytology has shown promise as a triage for HPV-positive women.[14] The simultaneous detection of the overexpression of p16, a cell-cycle arrest protein under normal physiologic conditions, and Ki-67, the cell proliferation marker, within the same cervical epithelial cell indicates HPV-induced deregulation of cell cycle.[15] A good to excellent reproducibility of this morphology independent biomarker has been observed with almost identical clinical performance of novice evaluations as compared with reference evaluations.[16] The p16/Ki-67 dual-stained cytology combines superior specificity and high sensitivity for detecting cervical intraepithelial neoplasia grade 2 or worse,[17, 18] which is now used as a triage strategy for managing the HPV-positive women during primary cervical screening,[19-21] for the women with abnormal Pap cytology,[22] and for the HPV-positive women with normal Pap cytology.[23]

There is increasing evidence that p16/Ki-67 dual-stained immunocytology (DS) can be used as an alternative test for triaging HPV-positive women.[24-26] Such a kind of DS tests can overcome the uncertainty of cytology through objective markers. In KPNC, ATHENA, and several other studies, DS test

has been shown to have better performance compared to cytology for the detection of CIN3+/CIN2+ in HPV-positive women.[27-32]

CINtec PLUS is one of the DS techniques specific to p16 and Ki-67, and its accuracy has been clinically and epidemiologically validated. CINtec PLUS has also been used as the comparator standard for evaluating various DS tests.[33, 34] It is a triage test for cervical cancer screening. It identifies underlying transforming HPV infections in women with abnormal Pap cytology results to send the women who will benefit most from colposcopy for follow-up testing. Currently, the CINtec *PLUS* Cytology test is included in guidelines in Ecuador, France, Germany, Hong Kong, Portugal, South Africa, and Spain.

This study aimed to conduct Human Papillomavirus (HPV) DNA testing and HPV 16/18 Genotyping based Cervical Cancer Screening among married women in Bogalay Township, Ayeyarwady Region, Myanmar and to identify the cervical cell abnormalities using liquid based cytology (LBC) and dual-biomarker p-16/Ki-67 immuno-cytology among HPV positive women.

MATERIALS AND METHODS

Study population and design

This study was a prospective cross-sectional descriptive study. This study was performed with the local administrative/health authorities in eleven wards of Bogalay Township, Ayeyarwady Region, Myanmar. From the community in each ward of Bogalay Township, about 60 married women aged between 30 to 55 years who want to take part in this study were recruited. Convenience sampling method was used. Cervical cancer screening was performed in the 100 bedded general hospital, Bogalay Township.

A total of 677 married residing in Bogalay Township, Ayeyarwady Region, Myanmar were screened in 2020. After obtaining a written informed consent, a thorough history was taken using structured-proforma. Then, speculum examination was performed under good light source. Cervical cells were obtained by sterile disposable cytobrush and collected in Cobas-cell-collection-media. Firstly, the collected samples were stored in 4°C refrigerator at 100 bedded general hospital, Bogalay Township. Then, those samples were sent to the Technology Development Division, Department of Medical Research (DMR), Yangon at room temperature within 6 hours. Those samples were stored in 4°C (at DMR) refrigerator prior to testing.

HPV DNA testing and HPV16/18 genotyping

HPV-DNA testing and HPV-16/-18 Genotyping were performed using cobas 4800 System.

Principles of the procedure

The cobas HPV test was based on two major processes: (1) automated specimen preparation to simultaneously extract HPV and cellular DNA; (2) PCR amplification of target DNA sequences using both HPV and β-globin specific complementary primer pairs and real-time detection of cleaved fluorescent-labeled HPV and β-globin specific oligo-nucleotide detection probes. The concurrent extraction, amplification and detection of β-globin in the cobas HPV test monitored the entire test process.

The master mix reagent for the cobas HPV Test contained primer pairs and probes specific for the 14 high-risk HPV types and β-globin DNA. The detection of amplified DNA (amplicon) was performed during thermal cycling using oligonucleotide probes labeled with four different fluorescent dyes. The amplified signal from 12 high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), was detected using the same fluorescent dye, while HPV16, HPV18 and β-globin signals were each detected with their own dedicated fluorescent dye.

Specimen preparation

Specimen preparation for the cobas HPV test is automated with the use of the cobas x 480 instrument. On the cobas x 480 instrument, collected specimens were digested under

denaturing conditions at elevated temperatures and then lysed in the presence of chaotropic reagent. Released HPV nucleic acids, along with the β-globin DNA serving as process internal control, were purified through adsorption to magnetic glass particles, washed and finally separated from these particles, making them ready for PCR amplification and detection.

PCR amplification

Target selection

The cobas HPV test used primers to define a sequence of approximately 200 nucleotides within the polymorphic L1 region of the HPV genome. A pool of HPV primers present in the master mix was designed to amplify HPV DNA from 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Fluorescent oligonucleotide probes bound to polymorphic regions within the sequence defined by these primers. An additional primer pair and probe targeted the human β-globin gene (330 bp amplicon) to provide a process internal control.

Target amplification

EagleZ05 DNA Polymerase, a chemically modified version of *Thermus* species Z05 DNA polymerase, was utilized for "hot start" amplification of the HPV targets and the β-globin control. First, the PCR reaction mixture was heated to activate Eagle Z05 DNA Polymerase, to denature the viral DNA and genomic DNA and to expose the primer target sequences. As the mixture cooled, the upstream and downstream primers annealed to the target DNA sequences. The EagleZ05 DNA Polymerase, in the presence of divalent metal ion and excess dNTPs, extended the primer(s), and a second DNA strand was synthesized. This completed the first cycle of PCR, yielding a double-stranded DNA copy of the target region of the HPV genome and β-globin gene. The DNA Polymerase extended the annealed primers along the target templates to produce an approximately 200-base pair double-stranded HPV target DNA molecule or a 330 base pair β-globin DNA molecule termed an amplicon. This process was repeated for a number of cycles, each cycle effectively doubling the amount of amplicon DNA. Amplification was occurred only in the region of the HPV genome and/or β-globin gene between the appropriate primer pair. The entire genome was not amplified.

Automated real-time detection

The cobas HPV test utilizes real-time PCR technology. Each oligonucleotide probe in the reaction was labeled with a fluorescent dye that serves as a reporter, and with a quencher that quenches fluorescent emissions from the dye in an intact probe. As amplification progressed, probes that were complementary to the amplicon bound to specific single-stranded DNA sequences and were cleaved by the 5’ to 3’ nuclease activity of the EagleZ05 DNA Polymerase. Once the reporter dye was separated from the quencher by this nuclease activity, it emitted fluorescence of a characteristic wavelength when excited by the proper spectrum of light. This characteristic wavelength for each dye allowed HPV-16 amplicon, HPV-18 amplicon, other HR amplicon (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and the beta-globin (internal control) to be measured independently because the probes specific for these sequences were labeled with different dyes.

Work flow

Firstly, collected specimens were thoroughly mixed in the vortex mixer to re-suspend the cells immediately prior to loading. Then, specimens were directly loaded in barcoded primary containers in cobas x 480 instrument. Amplification/detection reagents (HPV MMX and HPV Mg/Mn), Controls [HPV (+) C and HPV (–) C] and proteinase K [PK] were loaded directly onto the reagent carrier and scanned by the cobas x 480 instrument automatically.

The cobas 4800 Software tracked the use of the reagents and reagent reservoirs. The software also verified that sufficient reagents were loaded on the instrument. Sample preparation was started by clicking on “Start Run”. After successful completion of sample preparation, the plate carrier was unloaded

from cobas x 480 instrument. After sealing the microwell plate, the plate was transported to the cobas z 480 analyzer and the amplification and detection run was started. All assay and run validation is performed by the cobas 4800 Software.

For the NILM (≥ 30 years) population, the sensitivity and the specificity of the cobas HPV test for ≥CIN2 histology were 83.2% with (95% CI:75.9% to 88.6%) and 60.4% with (95% CI: 58.9% to 61.9%), respectively. The sensitivity and specificity of the cobas HPV Test for detecting ≥ CIN3 histology were 90.0% with (95% CI: 81.5% to 94.8%) and 60.0% with (95% CI: 58.5% to 61.5%), respectively.[35]

Liquid Based Cytology (LBC)

One ml of the samples was taken and added in 5ml tube and then centrifuged at 8000rpm for 3 minutes. Cell deposits were placed over the glass slide manually. For each subject, two cervical smears were made for LBC and immunocytology. Then, fixative spray was applied over the cervical smears.

For LBC, Papanicolaou (Pap) staining was done. Cytological screening was performed using Bethesda system. The Bethesda System stands out as a model of standardized reporting in cervical cytology. The system has five components of a Pap smear report-specimen type, adequacy, general category, interpretation, and adjunctive testing.[36]

Interpretation/result (Bethesda System)

(a) *Negative for intracellular lesions or Malignancy (NILM)* when there is no cellular evidence of neoplasia – whether or not there are organisms or other non-neoplastic findings.

(b) *Epithelial cell abnormalities*

Squamous cell abnormalities

1. Atypical squamous cells (ASC)

   Of undetermined significance ASC of undetermined significance

   Cannot exclude HSIL atypical squamous cells, cannot rule out HSIL (ASC-H)

2. LSIL (encompassing HPV/mild dysplasia/CIN 1)
3. HSIL (encompassing moderate and severe dysplasia/ CIS/CIN 2 and CIN 3)
4. With features suspicious for invasion (if suspected)
5. Squamous cell carcinoma.

Glandular cell abnormalities

1. Atypical
   Endocervical cells (NOS or specify)
   Endometrial cells (NOS or specify)
   Glandular cells (NOS or specify)
2. Atypical
   Endocervical cells (Favor neoplastic)
   Glandular cells (Favor neoplastic)
3. Endocervical Adenocarcinoma in situ
4. Adenocarcinoma
   Endocervical
   Endometrial
   Extrauterine
   NOS

Dual-biomarkers p-16 and Ki-67 immunocytology

It was performed using CINtec *PLUS* Cytology kit, Bench Mark GX IHC/ISH system, Ventana, USA. CINtec *PLUS* Cytology is the test that uses dual-biomarker technology to simultaneously detect p16 and Ki-67 to provide a strong indicator of the presence of transforming HPV infections. Positive p16/ Ki-67 dual-stained cells were characterized by a brown cytoplasmic/nuclear signal for p16 overexpression and a red nuclear signal for Ki-67 expression within the same cell. The presence of at least one double-stained cell was sufficient to score the sample as positive, regardless of the morphological appearance. All specimens were conducted per the manufacturer's instructions and stored at room temperature.[37]

Histopathology

HPV positive high-grade cervical lesions were followed by histopathology. Hematoxylin and Eosin staining were used. WHO Histopathological classification of

tumors of the uterine cervix (in brief) [38] are as follows;

(a) Normal uterine cervix
(b) Non-neoplastic lesions
(c) Squamous tumours and precursors

1. Benign squamous cell lesions
2. Cervical intraepithelial neoplasia (CIN)
   - CIN 1 - Condyloma
   - CIN 2
   - CIN 3
3. Squamous cell carcinoma

(d) Glandular tumours and precursors

1. Benign glandular lesions
2. Glandular dysplasia
3. Adenocarcinoma in situ
4. Microinvasive adenocarcinoma
5. Invasive adenocarcinoma

Data analysis

All study data were double-entered using Microsoft Excel. Statistical analysis of the data was performed using Statistical Package for Social Sciences (SPSS-16.0) (http:// en.softonic. com/s/ spss-16-full-version-free-download/).

RESULTS

A total of 677 married women residing in Bogalay Township, Ayeyarwady Region, Myanmar were screened in February 2020. Regarding the baseline characteristics of women, the mean age was 40 years (±SD,7.3 years: range-30-55), mean age of menarche was 14 years (±SD,1.6 years: range-11-19), and mean age of first marriage was 23years (±SD,5.5 years, range-14-46). Most of the screened women had single marriage (94.5%) but 5.5% had second marriage. In Parity, women who were less than parity three was (45.8%) (310/677) and equal or more than parity three was (54.2%) (367/677). Regarding the education status, (32.1%) (217/677) were graduated, (23.9%) (162/677) were high-school level, (16.7%) (113/677) were middle-school level, (23.2%) (157/677) were primary-school level, and (4.1%) (28/677) were illiterate.

HPV DNA testing and HPV-16/-18 genotyping

HPV was identified in 4.6% (31/677) of screened women in Bogalay Township, Aye-yarwady Region, Myanmar Fig. 1. comprising HPV-16 (1%), HPV-18 (0.3%) and pooled-12-other-HR-HPV-genotypes (3.3%).

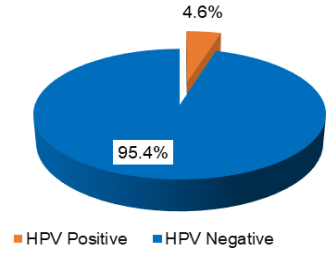

Fig. 1 Proportion of HPV infection among screened women residing in Bogalay Township, Ayeyarwady Region (n=677)

Among HPV positive cases, HPV-16 was 22.6 % (7/31), HPV-18 was 6.5% (2/31) and pooled-12-other-HR-HPV-genotypes (HPV-31,-33,-35,-39,-51,-52,-56,-58,-59,-66 and -68) were 70.9%(22/31) Fig. 2.

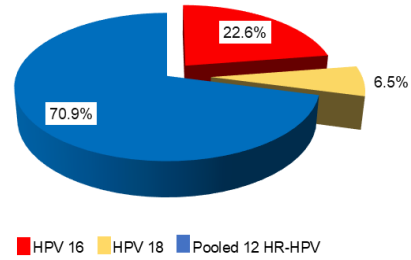

Fig. 2. Proportion of HPV genotypes among HPV infected women residing in Bogalay Township, Ayeyarwady Region (n= 31)

Liquid Based Cytology (LBC)

Among HPV positive cases, 87.1% (27/31) had cervical cytological abnormalities comprising 16.1% (5/31) had high-grade squamous intraepithelial lesion (HSIL), 48.4% (15/31) had low-grade squamous intraepithelial lesion (LSIL) and 22.6% (7/31) had atypical squamous cells of undetermined significance (ASCUS). Only 12.9% (4/31) were negative for intraepi-thelial lesion or malignancy (NILM) Fig. 3.

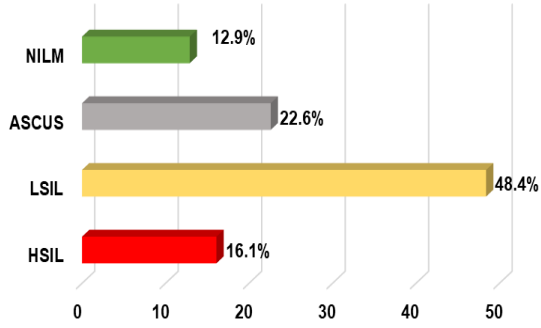

Fig. 3. Proportion of cervical cytological abnormalities among HPV positive screened women residing in Bogalay Township, Ayeyarwady Region (n= 31)

Histopathology diagnosis

In histopathology diagnosis of HPV-positive/HSIL women, 40% had cervical intraepithelial neoplasia (CINII), 40% (CIN III) and 20% (invasive squamous cell cancer of cervix) Fig. 4.

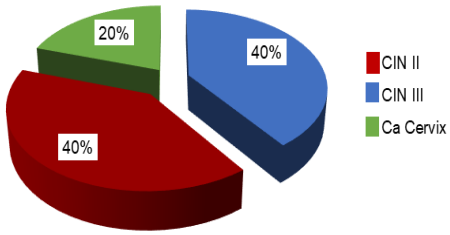

Fig. 4. Proportion of histopathology diagnosis among HPV-positive/HSIL women residing in Bogalay Township, Ayeyarwady Region (n=5)

Table 2. Detection of dual-biomarker p-16/ Ki-67 immunocytology among HPV positive screened women residing in Bogalay Township, Ayeyarwady Region

LBC	Dual-biomarker p-16/Ki-67 immunocytology		Total (%)
	Positive (%)	Negative (%)	
NILM	0	4 (100)	4 (100)
ASCUS	1 (14.3)	6 (85.7)	7 (100)
LSIL	10 (66.7)	5 (33.3)	15 (100)
HSIL	5 (100)	0	5 (100)
Total	21	10	31

Dual-biomarker p-16 and Ki-67 immunocytology

In immunocytology of HPV-positive women, all HSIL (100%) cases, 66.7% of LSIL, 14.3% of ASCUS was positive with both p-16 and Ki-67 immuno-biomarker but negative in NILM cases (Table 2 & Fig. 5).

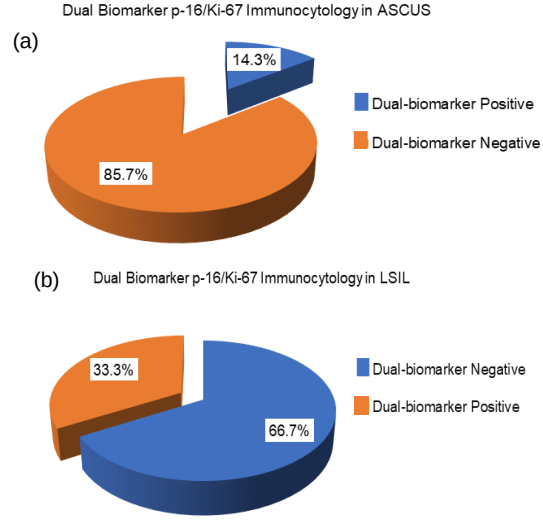

Fig. 5. Detection of dual-biomarker p-16/Ki-67 immunocytology among HPV positive women with (a) ASCUS and (b) LSIL in LBC

DISCUSSION

The goal of HPV-based cervical cancer screening is to avoid misdiagnosis and the over- or under- treatment it causes. While Pap cytology has had a positive impact on HPV infection and cervical cancer screening over the past 50 years, it is resource-intensive and subject to misinterpretation and missed disease. Screening women for the presence of HPV is a critical aspect for secondary prevention and early treatment of cervical cancer. Regarding the prevalence of HPV among women with normal cervical cytology in Asia widely varied even in the single region: 1.7%-45.6% in China, 2.3%-36.9% in India, 7.6% in Bangladesh, 3.3%-40.6% in Thailand, 1.5%-10.2% in Vietnam, 3.1% -46.7% in Malaysia, 9.3% in Philippines, 8.8%-31% in Indonesia.[1] In ATHENA HPV study i.e, multicenter US cervical cancer screening trial, prevalence of HPV was

16.4% in which pooled-12-HR-HPV, HPV-16 and HPV-18 were detected in 12.6%, 2.8% and 1.0% of women respectively.[39]

In Myanmar, Mu Mu Shwe, *et al* investigated the HPV-based cervical cancer screening in general population in which HPV was detected in 5.5% of women residing in North Okkalapa Township, Yangon in 2017,[40] 10% of women attending gynaecology clinics in two selected-hospitals, Yangon in 2018-2019[41] and 6.1% of women residing in Magway region 2019[42]. In the present study, HPV was detected in 4.6% of women in Bogalay Township, Ayeyarwady Region. Prevalence of HPV infection among women in this study was relatively lower than the previous studies. Variation of HPV prevalence in general population is similar with other Asia studies.

HPV-based cervical cancer screening in Myanmar indicated that about 4%-10% of HPV positive women in general population were needed to attend regular follow up and further management. If there had the resources for HPV 16/18 genotyping, the number of cases for colposcopy and biopsy would have been reduced. According to this study, only 1.3 % of screened women i.e, HPV16/18 positive cases will be needed for colposcopy and biopsy. Therefore, the burden of health care providers for further management will also be reduced. In rural areas of Myanmar, there are low human resources for primary health care. It will be one of the major obstacles to achieving the success of National cervical cancer screening program in Myanmar.

Worldwide, prevalence of HPV 16/18 in women with normal cervical cytology, precancerous cervical lesions and invasive cervical cancer were 3.9%, 25.8%, 51.9% and 69.4%.[1] In Myanmar, Win Win Mya (2003) reported that, HR-HPV was detected in 81.2% (13/16) of cervical tissue with SCC, 5.56% (2/36) with CINI and 8.33% (3/36) with normal cytology and HPV-16 was the most common genotype followed by HPV-18.[43] Regarding the prevalence of HPV in cervical pre-cancer and cancers, Mu Mu Shwe, *et al* determined that, HR-HPV was identified in CINI (39.3%), CINII (58.6%), CINIII (66.7%), and SCC (77.1%) in 2012-2014. In CINI, the most common genotypes were HPV-16 (69.7%) followed by HPV-31 (21.2%), HPV-18(6.1%) and HPV-58 (3.0%). In CINII, HPV-16 was most commonly detected (52.9%) followed by HPV-31 (23.5%), HPV-58 (17.6%), and HPV-18 (5.9%). In CINIII, HPV-16 was also the most common genotype (85.7%) followed by HPV-31 (7.1%) and HPV-58 (7.1%).[44]

In another study in 2016 reported that HPV infection and genotypes among cervical cancer cases; HPV was identified in 85.8% of cervical cancer patients attending Central Women Hospital, Yangon. The most prevalent HPV genotype was HPV-16 (63.7%) and HPV-18/45 (17.6%).[45] Vaccine preventable genotype, HPV-16 was the most common genotype among precancerous cervical lesions and invasive cervical cancer in Myanmar.

Regarding the HPV genotypes among HPV-positive cases in general population, HPV-16, HPV-18 and pooled-12-HR-HPV types (HPV-31,-33,-35,-39,-51,-52,-56,-58,-59,-66 and-68) were 25%, 8.3% and 66.7% respectively in North Okkalapa Township, Yangon (2017),[40] 32%, 18% and 50% respectively in two selected-hospitals, Yangon (2018-2019)[41] and 12.5%, 12.5% and 75% respectively in Magway (2019).[42] In this present study, HPV-16 was 22.6 %, HPV-18 was 6.5% and other-HR-HPV-genotypes were 70.9%. HPV types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide. This finding indicated that, among HPV positive cases in general population, HPV-16/-18 genotypes were 25%-50%. Those HPV positive cases among general population were high risk women who may develop cervical cancer.

In previous study, among HPV positive cases in two selected-hospitals, Yangon (2018-2019), 95.5% (21/22) had cervical cytological abnormalities comprising 27.3% (6/22) had high-grade squamous intraepithelial lesion (HSIL), 45.5% (10/22) had low-grade squamous intraepithelial lesion

(LSIL) and 22.7% (5/22) had atypical squamous cells of undetermined significance (ASCUS).[41] The present study found out that among HPV positive cases, 87.1% (27/31) had cervical cytological abnormalities comprising 16.1% (5/31) had high-grade squamous intraepithelial lesion (HSIL), 48.4% (15/31) had low-grade squamous intraepithelial lesion (LSIL) and 22.6% (7/31) had atypical squamous cells of undetermined significance (ASCUS). Both studies showed consistent findings.

Dual stain for p16/Ki-67 is a kind of technique using immunohistochemistry specific to p16 and Ki-67, respectively. The p16, a cyclin-dependent kinase inhibitor, acts as a tumor suppressor in most cells,[46] but HPV E7 oncoprotein mediates the degradation of retinoblastoma protein (Rb), and p16 exhibits oncogenic activity in HPV-transformed cervical cancer cells.[47] Ki-67 is a positive marker for cell proliferation.[48] In normal conditions, they usually do not co-express in the same cervical epithelial cell. Co-expression of two molecules indicates the deregulation of the cell cycle mediated by infected high-risk HPV and suggests the possibility of high-grade CIN.[49] There is increasing evidence that p16/Ki-67 dual-staining immunocytology can be used as an alternative biomarker, showing overall high sensitivity and specificity for identifying high-grade CIN and cervical cancer.

The positive p16/Ki-67 dual-staining is associated with HR-HPV infection, particularly with HPV 16 and 18.[50-52] The p16/Ki-67 positive rate in the HPV-positive women was 78.9%, significantly higher than 9.4% in the HPV-negative patients.[53] The association of p16/Ki-67 positivity with HPV16 and/or 18 infections was 2-4 folds stronger compared to the cases infected with other HR-HPV types.[50, 51]The positivity of p16/Ki-67 dual-staining also strongly indicates CIN2+ or high-grade squamous intraepithelial lesion (HSIL). The positive rates of p16/Ki-67 dual-staining in HR-HPV positive women with diagnoses of negative for intraepithelial lesion or malignancy (NILM), ASCUS, LSIL, atypical squamous cells cannot exclude HSIL (ASC-H), and HSIL were 3.0%, 23.6%, 25.8%, 78.6%, and 100.0%, respectively.[53] The positive rate increased from 31% in women with negative cytology to 92% in women with HSIL.[50] Similarly, the positive rate of p16/Ki-67 in women with CIN3 was 86%, which was significantly higher than 24% in women without biopsy results.[54] All patients with cervical cancer showed double staining positive for P16/Ki-67.

In the present study, the positive rates of p16/Ki-67 dual-staining in HR-HPV positive women with diagnoses of negative for intraepithelial lesion or malignancy NILM, ASCUS, LSIL and HSIL were 0%, 14.3%. 66.7% and 100% respectively. This study found out that the CINtec positivity rates were higher in cytological result of higher severity. Some studies investigated that the positive rate of p16/Ki-67 increased significantly with the severity of cytological and histological abnormalities.[50, 51, 55, 56] This study was consistent with those above studies. Angela L Abreu, *et al* (2021) investigated the validation of CINtec PLUS cytology kit in the diagnosis of persistent HPV infections which revealed that CINtec positivity rates were expectably higher in women with cytological result of higher severity -23% for NILM, 34% for ASCUS, 45% for LSIL, 79% for ASCH/ HSIL.[57] However, CINtec positivity in the present study was not determined in NILM cases.

The sensitivity and specificity of p16/Ki-67 dual-staining for CIN2+ were 74.9%-90.9% and 72.1 %-95.2 %, respectively .[51, 55, 56, 58] The positive rate of CIN2+ detected by p16/Ki-67 dual-staining was 92.7%, which was more sensitive than 71.1% by HPV16/18 genotyping alone.[50] Compared with HPV detection, p16/Ki-67 dual-staining has higher specificity in detecting CIN2+ and can significantly reduce the number of patients referred to colposcopy, especially for young women with high HPV infection rate.[55, 56] In the present study, histological diagnosis of p16/Ki-67 dual-stain positive in HPV positive/HSIL women was

CIN2+ (100%). This study determined that dual-biomarker p-16 and Ki-67 immunocytology was very useful to confirm the high-grade cervical lesions of HPV positive women.

Therefore, p16/Ki-67 dual-staining immunocytology is of great significance in screening and triaging of cervical cancer and pre-cancerous lesions. It could lead to improved clinical outcomes. Various combinations of screening and triage tests can result in differences in disease detection and the number of colposcopies required. A strategy that results in more disease detection without increasing the colposcopy rate can aid clinicians making decisions that result in improved clinical outcomes and lower healthcare costs.

Conclusion

This study highlighted that dual-biomarker p-16 and Ki-67 immunocytology was very useful to confirm the high-grade cervical lesions of HPV positive women. In addition, triage with this dual-biomarker immune-cytology among low-grade cervical lesions of HPV positive women reduce the number of women that require referral to colposcopy. Results in more disease detection without increasing the colposcopy rate can aid clinicians making decisions.

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Competing interests

The authors have declared that no competing interest exists.

Ethical consideration

This study was approved by Institutional Review Board (IRB), Department of Medical Research, Yangon. IRB No/Approval No: 2019-74/Ethics/DMR/ 2019/ 081AE/2020

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