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ORIGINAL ARTICLE

Benefits of P16/Ki- 67 Cytology as a Co-Test for High-Risk HPV-Positive Women in a "See and Treat Strategy"

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ABSTRACT

Background: While the high-risk human papilloma virus DNA (hr-HPV-DNA) test is the primary tool for cervical cancer screening, with visual inspection with acetic acid (VIA) serving as a triage test where Pap cytology is not available, the low inter-observer agreement associated with VIA means that its reliability is limited and a more efficient test is still required. The aim of this study was to compare the performance of p16/Ki-67 cytology with that of VIA in the detection of cervical precancer and its feasibility as an alternative triage method in the "see and treat strategy".

Methods: In this hospital-based cross-sectional study, we utilised stored, provider-collected specimens from a previous study involving women referred with cervical abnormalities to a tertiary hospital in Kisumu County, Kenya. The samples were analysed using both Xpert testing and p16/Ki-67 dual staining. All high-risk HPV–positive women with cervical lesions were triaged using VIA and p16/Ki-67 cytology. Cervical intraepithelial neoplasia grade 2 or worse (≥CIN2) was defined as the clinical endpoint.

Results: Compared with VIA, p16/Ki-67 immunostaining had a significantly greater sensitivity (84.6% vs. 59.0%), specificity (44.0% vs. 62.0%), positive predictive value (28.2% vs. 28.8%) and negative predictive value (91.7% vs. 85.3%).

Conclusion: p16/Ki-67 immunostaining for the detection of ≥CIN2 demonstrated high sensitivity and a high negative predictive value in our study, consistent with findings from several previous studies. These results suggest that the assay is superior to VIA for identifying ≥CIN2 and could serve as an alternative tool for triaging women who test positive for primary HPV within the current "see-and-treat" strategy.

BACKGROUND

The incidence and mortality of cervical cancer (CC) continue to rise despite every effort being made to contain the disease, leading to major global health imbalance that requires urgent attention from all stakeholders.[1] CC ranks 8th in incidence among women worldwide, with an estimated 660,000 new cases and 348,000 deaths annually.[2] The disease burden is more pronounced, especially in sub-Saharan Africa (SSA), owing to the high prevalence of HIV in the region [3]; more so in Eastern Africa, where the age-standardised incidence estimate is 40.0 per 100,000 women.[4]

Uterine cervical intraepithelial neoplasia (CIN) is caused primarily by persistent infection with human papilloma virus (HPV), especially types 16 and 18.[2] The virus penetrates the basal layers of epithelial cells through micro-abrasion of the transformation zone of the cervix via two oncogenes, E6 and E7 [5], which also interact with and inhibit various cell cycle-regulating proteins, such as the retinoblastoma gene product pRB and the p53 protein, resulting in cervical dyskaryosis following proteolytic degradation. Increased expression of these viral oncogenes in dysplastic cervical cells is reflected by increased coexpression of p16INK4a and Ki-67.[6] Currently, high-risk human papillomavirus DNA (hr-HPV-DNA) testing serves as the primary screening tool for CC. However, in settings where Pap cytology is unavailable, visual inspection with acetic acid (VIA) is used as a triage test [7], although its reliability is limited by low intra-observer agreement. Consequently, the integration of VIA examination in a treatment plan should be performed with much caution given that the technique is largely based on observer qualitative judgment [8], experience and examination environment. These factors often vary depending on facility infrastructure and regional resources, leading to a high rate of false positivity and low specificity.[9] Moreover, immunostaining for p16/ki-67 has been shown to be more efficient in identifying CIN in a number of studies and can serve as an alternative triage test for all HPV-positive women.[10,11] Compared with traditional VIA, p16/Ki67 cytology is a more promising alternative for rapid, low-cost, and high-volume HPV testing in low- and middle-income countries (LMICs) [12], especially with consistent efficacy, as shown in a number of clinical trials.[11,13]

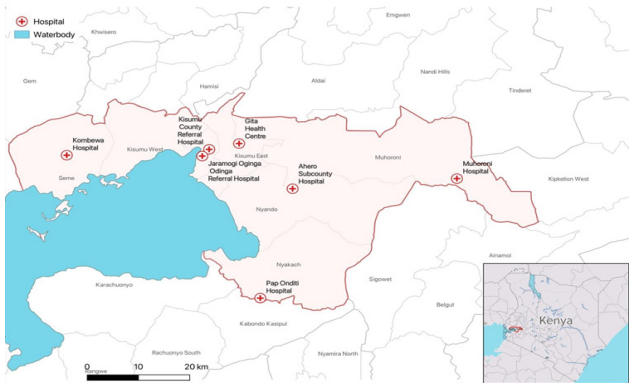
The dual-stain p16INK4a protein is derived from the host p16INK4a/CDKN2A tumour suppressor gene found at chromosome 9.[14] In humans, this cytoplasmic antigen has been identified as a biomarker for transforming HPV infection and therefore can be used as a surrogate marker of hr-HPV infection.[14] Additionally, Ki-67 (MIB-1) is a nuclear antigen proliferative biomarker that is highly expressed in HPV-infected mature squamous cells. These usually manifest as a proliferating cell; hence, they are closely linked to tumours of the cervix.[10] Overall, p16 acts as a cell cycle regulatory protein that induces cell cycle arrest, whereas ki-67 functions as a cell proliferation marker. Under normal physiological conditions, these two biomarkers are rarely co-expressed in the same cervical epithelial cells.[6] Thus, the co-expression of these two molecules in cervical cells suggests that the deregulation of the cell cycle is mediated by hr-HPV infection, which predicts the presence of high-grade cervical intraepithelial lesions (HSIL) [15]. The integration of the p16ink4a / ki-67 biomarker as a point-of-care test to be used specifically to identify cases of cervical dysplasia with subsequent treatment at one visit is an option currently being explored in a number of developing countries [16], especially with the evolving landscape of HPV vaccination and changes associated with HPV genotype prevalence.[17] The results from a recent Rwanda screen, notify, see, and treat CC screening programme revealed that loss to follow-up owing to VIA examination scheduling was a key challenge in determining the optimal uptake in sub-Saharan Africa and the need for an alternative.[18] Locally, attempts to evaluate the performance of p16/Ki-67 cytology ended inconclusively owing to inadequate sample size.[19,20] In this study, we leveraged referrals from peripheral facilities with cervical abnormalities to guarantee adequate sample size.

MATERIALS AND METHODS

Study Area

This study was performed at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) in Kisumu County between the years 2021 and 2023 (Figure 1). The choice of Kisumu County was based on available data for CIN from the previous study and the endemicity of HIV in the region[21], which also serves as a potential enhancer for HPV persistence in women infected.

FIGURE 1: Map of Kisumu County



Study Design and Sampling Procedures

We utilised stored provider-collected samples from 189 hr-HPV-positive women with colposcopic biopsies who participated in a previous parent cross-sectional study.[22] The parent study protocol provides details of study population, consent approval, sample size determination, and inclusion and exclusion criteria. Briefly, a total of 517 women aged 25 to 65 years with cervical abnormalities referred from peripheral facilities in Kisumu County, Kenya, were enrolled after obtaining their written consent and research ethics approval.

Sample Size Estimation

A total of 4465 females were examined for CC at JOOTRH in 2020.[23] The minimum sample size was estimated using the formula by Yamane (1967), expressed as: Sample size (SS) = N / [1 + (N × e^2)], where N represents the population size and e is the level of precision.

$$n = \frac{4465}{1 + (4465 \times 0.05^2)} = 367.1$$

The calculated minimum sample size was approximately 367 participants.

Sample Adjustment

Based on previous experience from a study in similar setting[20], a 30% adjustment was applied to account for potential dropout, following the approach described by Charan Singh and others. (2021).[24] The adjusted sample size (N_1) was calculated using the formula $N_1 = n / (1 - d)$, where n represents the required sample size (367) and d the anticipated dropout rate (0.3). This resulted in an adjusted sample size of 524 participants. However, a total of 517 participants were ultimately enrolled, which was still considered adequate for the study.

Inclusion and Exclusion Criteria

Eligibility was defined in the parent study protocol[22] and included women with a history of sexual activity, those who had an initial positive result on visual inspection with acetic acid (VIA) from referral facilities, and those who provided informed consent. Women were excluded if they were pregnant, had used vaginal medication within two days prior to the screening, or presented with a history of hysterectomy, muco-purulent discharge, or active vaginal bleeding.

Ethical Approval

The study protocol received ethical approval from JOOTRH (ERC-IB/VOL.1/602). All participants provided written informed consent before enrolment. Those intending to withdraw did so at any time. The participants' privacy and confidentiality were upheld. All study participants were provided with free health education on cervical cancer. Arrangements were made to refer participants tested positive for malignant and premalignant lesions to the comprehensive care centres at JOOTRH for treatment, care and support.

Clinical Evaluation of Cervix Using Colposcopy and VIA

Clinical examination involved a gynaecological examination with inspection of the cervix uteri and collection of specimens by a gynaecologist in a separate room. In this procedure, a gynaecologist sequentially

evaluated the cervix via the 5% acetic acid to assess the presence of abnormal blood vessels and cervical lesions within the squamocolumnar junction. A cervix stained with acetic acid within the squamocolumnar junction, together with capillaries appearing as red spots, was considered abnormal and suggestive of a premalignant lesion. This was followed by cervical sample collection in PreservCyt® Solution (Hologic) via CervexBrush® (Rover) for HPV DNA and p16/ki-67 cytology tests as described in the parent study protocol [22] and in the recruitment flowchart (Figure 2).

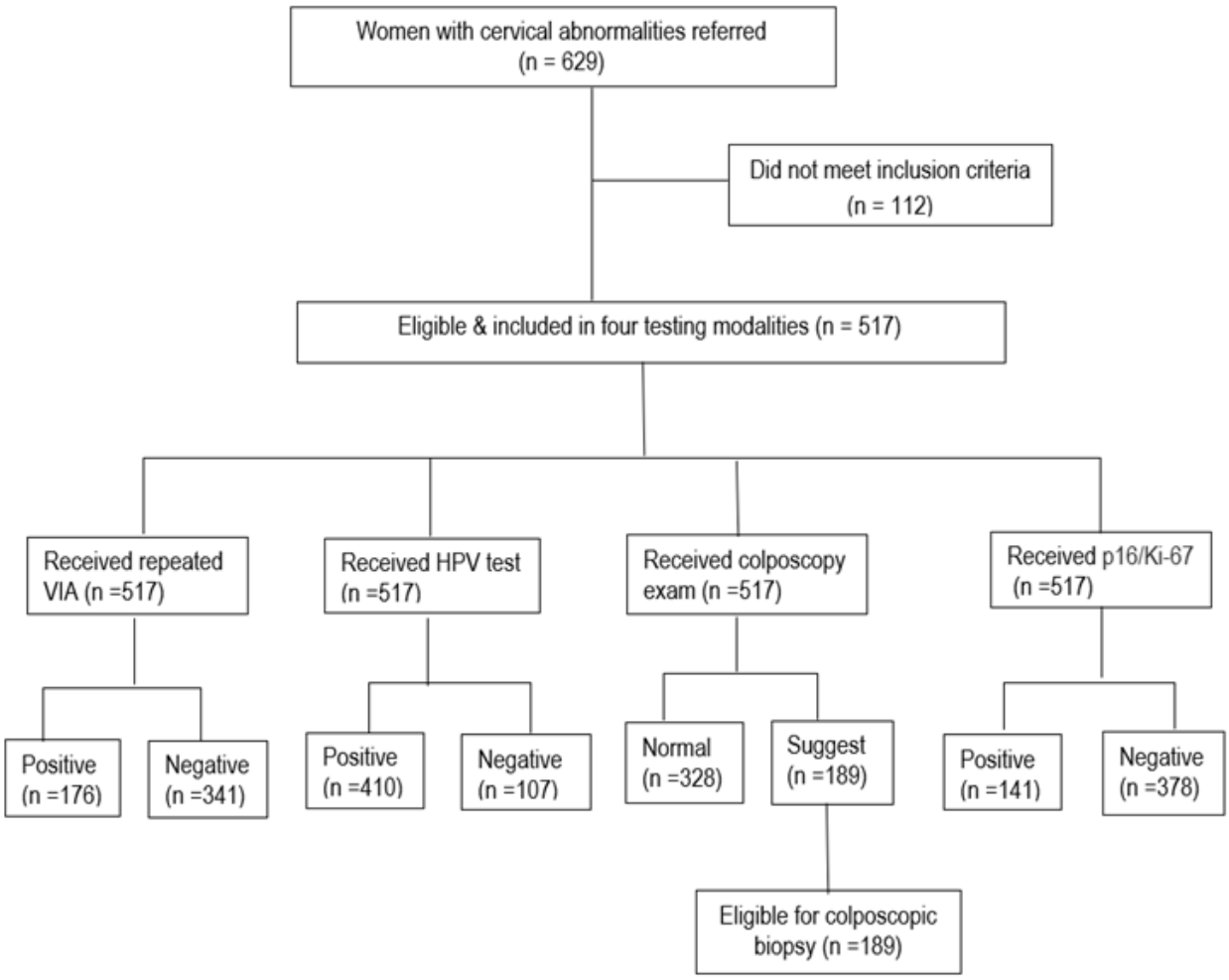
Laboratory Procedures

HPV-DNA testing: This was performed according to the manufacturer's instruction.[25] HPV DNA was tested via Gene Xpert® HPV (Cepheid, Sunnyvale, California, United States [US]) by transferring a 1-mL aliquot of a cervical sample directly into an Xpert cartridge containing

DNA extraction reagents and primers with probes for amplification and HPV detection. Xpert HPV is based on a multiplex real-time PCR targeting E6 and E7 oncogenes of 14 hr-HPV genotypes. The amplification was conducted in five fluorescent channels, namely HPV16, HPV18/45, HPV31/33/35/52/58, HPV51/59, and HPV39/56/66/68, and the results were interpreted via Xpert software version 4.8 (Cepheid). HPV positivity was defined if the cycle threshold (CT) cutoff was ≤ 40 for HPV16 and HPV 18/45, and ≤ 38 for HPV31/ 35/33/52/58, HPV51/59, and HPV39/68/56/66. Samples were considered adequate if hydroxymethylbilane synthase was ≤38 cycle threshold.

p16ink4a / ki-67 Immunostaining test: The remaining residues of the PreservCyt specimen after HPV-DNA tests were further subjected to the ThinPrep 2000 Processor (Hologic Inc.) to prepare ThinPrep slides, which were then manually stained with CINtec PLUS p16/Ki-67® Roche MTM Laboratories according to manufacturer instructions [20]. After primary staining, the slides were further incubated with ready-to-use secondary reagents comprising (a) a polymer reagent conjugated with horseradish peroxidase (HRP) and goat anti-mouse.

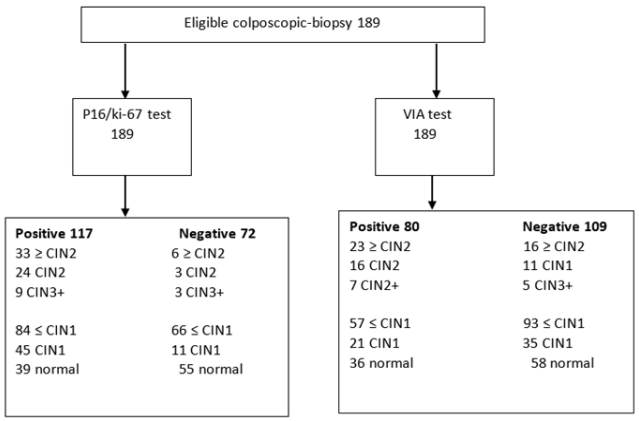
FIGURE 2: Participants' Enrolment Flow Chart



Fab'antibody fragments and (b) a polymer reagent conjugated with alkaline phosphatase and goat anti-rabbit Fab' antibody fragments. After counterstaining with alcohol-free haematoxylin, a 2-step mounting procedure was applied using an aqueous mounting medium, followed by a permanent mounting medium.

The test was performed in the histopathology laboratory of JOOTRH. Interpretation of p16/Ki-67 cytology slides was performed by two cytologists who reviewed all cases for the presence of (double immune-reactive cells) 1 or more cervical cell(s) showing an evident brown cytoplasmic and a red nuclear staining indicative of p16 and Ki-67 coexpression, respectively, for a positive result. The same slides were scanned and shared with a specialist pathologist at MOI Teaching and Referral Hospital for further review. High-grade squamous intraepithelial lesion (HSIL) slides were used as a positive control for each immunoreaction test.

FIGURE 3: The Two Tests Comparison Flow Chart



Histology test

Briefly, colposcopic biopsies fixed in 10% formaldehyde were processed via an automated tissue processor (HistoCore PELORIS 3; Leica Biosystems) via dehydration, clearing and infiltration in paraffin wax before being sectioned into 4 µm thick sections on clean slides. The prepared slides were then reloaded into the system for dewaxing in an oven, clearing in 2 changes of xylene and hydrated in alcohol in a descending grade of concentration. This was followed by staining in haematoxylin followed by eosin, then dehydration in ascending grades of alcohol. Finally, the stained slides were mounted in DPX, examined under power X10 and X40 microscopes, and scanned and shared with two different pathologists for review and reporting. Histopathology findings were classified according to the CIN scale: as normal, CIN grade 1, grade 2, grade 3 or invasive cancer.

All HPV test-positive women with suggestive colposcopy (189), including 80 (42%) VIA-positive and 109 (58%) VIA-negative women and 117 (62%) p16/ki-67-positive and 72 (38%) p16/ki-67-negative women, underwent biopsies (if a cervical lesion was present) for disease status verification as indicated in the comparison flowchart (Figure 3). All women were informed about the VIA result immediately, but for laboratory results, they were booked to collect after one month in the next clinic review visit. Cervical biopsies were read by two pathologists at JOOTRH for study quality assurance. Participants who were HPV-positive and VIA-positive or p16/ki-67-positive and eligible for thermal ablation, including those with cervical intraepithelial neoplasia grade 2 or 3 (≥CIN2) on biopsy, were treated according to the Kenyan national cancer treatment guideline.[7]

Data Analysis

The number of p16/Ki-67 immunostain-positive cells and ≥CIN2 according to VIA examination results were compared. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the two test modalities were calculated using SPSS software (version 25.0). Estimates were provided with 95% CIs. The significant differences in sensitivity, specificity, PPV, and NPV were calculated using the exact McNemar $\chi 2$ test. Statistical significance was defined as $p < .05$. All statistical tests performed were two-sided.

Ethics Approval and Consent to Participate

The institutional review board of JOOTRH provided ethics approval of the study number ERC.IB/VOL.1/602. Written informed consent was obtained from all participants.

RESULTS

Performance of P16/ki-67 Cytology and VIA

Among the 189 samples tested, 80 (42%) and 117 (82%) were CIN positive on VIA and p16/ki-67, respectively. There were 39 patients with pathologically confirmed ≥CIN2, including 27 patients with CIN2 and 12 patients with ≥CIN3. There were 150 cases defined as CIN1, including 56 cases of CIN1 and 94 cases of normal pathology as shown in Table 1. The sensitivity, specificity, PPV and NPV of p16/Ki-67 cytology and VIA for the detection of ≥CIN2 are shown in Table 2. The sensitivity of p16/Ki-67 cytology was 84.6%, which was significantly greater than that of VIA examination (59.0%, $P = .024$). The specificities of p16/Ki-67 cytology and VIA examination were 44.0% and 62.0%, $P = .003$), respectively. The PPV of p16/Ki-67 dual-stain was 28.2%, which was comparable to that of VIA examination (28.8%, p = .251). The NPV of p16/Ki-67 dual-staining was 91.7%, which was significantly greater than that of VIA examination (85.3%, $P = .002$).

TABLE 1: CIN Positivity Rate by p16/ki-67 and VIA Tests

Variable	N	Overall N = 189[1]	Colposcopic biopsy Result CIN1 & Normal N = 150[1]	CIN2 N = 27[1]	≥CIN3 N = 12[1]
VIA	189				
negative		109 (58%)	93 (62%)	11 (41%)	5 (42%)
positive		80 (42%)	57 (38%)	16 (59%)	7 (58%)
p16/ki67	189				
negative		72 (38%)	66 (44%)	3 (11%)	3 (25%)
positive		117 (62%)	84 (56%)	24 (89%)	9 (75%)

[1]n; numbers, (%): proportions. VIA: visual inspection with acetic acid, CIN: cervical intraepithelial neoplasia 1,2, or 3.

TABLE 2: Performance of P16ink4a/Ki-67 and VIA Tests to Detect ≥CIN2

	P16/ki-67 test		VIA test		
	n/No.	%(95%CI)	n/No.	%(95%(CI)	P value
Colposcopic-biopsy					
Sensitivity	33/39	84.6 (69.5 – 94.1)	23/39	59.0 (42.1 – 74.4)	.024
Specificity	66/150	44.0 (35.9 – 52.3)	93/150	62 (53.7– 69.8)	.003
PPV	33/117	28.2 (21.7 – 36.3)	23/80	28.8 (23.1 – 34.5)	.251
NPV	66/72	91.7 (87.8 – 94.7)	93//109	85.3 (80.0 – 89.7)	.002

≥CIN2, cervical intraepithelial neoplasia grade 2 or worse; 95%CI, 95 percent confidence interval; % proportions. VIA, visual inspection with acetic acid; PPV, positive predictive value; NPV, negative predictive value.

DISCUSSION

In an effort to eliminate cervical cancer worldwide, the World Health Organization (WHO) recently revealed new screening and treatment guidelines intended to accelerate and promote faster and better intervention outcomes as a matter of public health priority in what was termed the "see and treat" strategy.[2] Among the recommendations in the guidelines is the implementation of DNA-based HPV testing as a primary screening tool, which many countries, including Kenya, have adopted by gradually transitioning from Pap cytology testing to HPV-based screening or co-testing.[7] To date, major strides have been realised following the adoption of HPV-DNA testing as a primary tool with subsequent triage using VIA where Pap cytology is not available.[7] Despite these milestones, the low intra-observer agreement associated with VIA has been noted in a number of studies locally [19,20], and in some parts of Africa [8], highlighting a call for a more efficient alternative triage tool. In our study, only 20.6% (39/189) of all high-risk HPV-positive women who underwent colposcopy and biopsy had ≥CIN2, as shown in figure 3, suggesting that the low specificity associated with HPV-DNA tests could lead to a high referral rate for colposcopy and cervical biopsy, as reported earlier [10,26]. Consequently, the need to develop a more efficient triage test for hrHPV-positive women in order to reduce unnecessary colposcopic referrals can never be overemphasised.

In this study, we compared the performance of p16/Ki-67 cytology and VIA in the triage of primary HPV screening-positive women. For the detection of cervical intraepithelial neoplasia 2 and above (≥CIN2), the sensitivity of p16/Ki-67 cytology was significantly greater than that of VIA examination (84.6% vs. 59.0%, P = .024), whereas the specificity appeared significantly lower than VIA examination (44.0% vs. 62.0%, P = .003, respectively). The majority of the previous studies conducted on p16/Ki-67 cytology for the prediction of ≥CIN2 yielded similar sensitivity, thus supporting its potential as a promising and efficient triage test for high-risk HPV-positive women [10,11,26]. The low specificity of p16/Ki-67 cytology reported in this cohort may be attributed to the absence of HPV-negative women in the study. Including HPV negative women would more likely increase the number of healthy women with a low probability of a positive p16/Ki-67 or ≥CIN2 result. This implies that implementing p16/ki-67 cytology as a co-test for HPV-positive women is only suitable on the basis of sensitivity and negative predictive value but not on specificity, which still requires further evaluation with a homogenous population of women infected and uninfected with HPV. If fully validated, utilisation of p16/ki-67 cytology in cervical cancer screening may serve as an alternative triage test to the existing strategies for women with cervical-vaginal abnormalities[27] given

that the current approaches, including repeat cytology, HPV testing, and colposcopy-guided biopsy are facing challenges such as high referral rate [19]. However, the cost implication of the test is not yet estimated and may need to be considered prior to implementation.

Meanwhile, in a systematic review conducted earlier in the same study [27], the reported test performance and the receiving operating characteristics curves obtained from several large-scale longitudinal and prospective studies all agreed that implementing the p16/ki-67 assay as a triage for HPV-positive women to be used at one visit with subsequent treatment is feasible. Similar investigations from China, Romania, Singapore, Slovenia and Denmark analysing the performance of p16/ki-67 cytology with HPV-DNA test, Pap cytology or colposcopy recorded a higher sensitivity range of 66.0% - 96.7% and specificity range of 51.6% - 93.0% for p16/ki-67 than the other tests, with a sensitivity range of 42.1% - 85.7% and a specificity range of 14.7% - 95.2% [28,29]. In addition to its potential as an alternative co-test for primary HPV-positive women, the assay can also be useful in predicting the regression or progression of CIN2 [16], and serve as a triage tool for younger women aged ≤30 years who present with ASC-US or LSIL.[30] Although variable sensitivities have also been reported in some published research[26], this study maintains that the observed variation could be due to some technical problems caused by insufficient cellularity [11], including variation in study populations. In this study, we included a subgroup of women referred for abnormal cytology, thus explaining the relatively lower specificity observed both in p16/ki-67 cytology and VIA examination. Moreover, both tests had lower but similar positive predictive value (PPV) for the detection of high-grade ≥CIN2 (28.2% vs. 28.8%, P =.251, respectively), although p16/ki-67 had a significantly greater negative predictive value than VIA did (91.7% vs 85.3%, P = .002), which was comparable to the findings of the previous studies[10,11,31,32], which all revealed a reduced PPV with increased NPVs.

With the ever-increasing CC screening coverage through vast community outreach, the beauty of home-based self-sample collection for the primary HPV-DNA testing can never be overemphasised considering the cost associated with provider-collected samples and the challenge of limited resources experienced by many LMICs [3]. Despite these advantages, including preferences by the majority of female participants, experts have also argued that the cellularity of cervical cells in self-collected samples is limited and may lead to low sensitivity and reliability, particularly with the p16/ki-67 assay, where sample residues from primary HPV testing are preferred for convenience.[11] Consequently, women testing hr-HPV positive on self-collected samples may still be required to revisit their clinicians for additional provider-collected cervical smears, thus posing a challenge to this immunostaining technology [11] and to the model of a "see and treat programme" [18], particularly in LMICs where same-day treatment decisions are often necessary after primary screening to reduce patient loss-to-follow-up.[33]

Limitation of the study

The participants were recruited from a gynaecological clinic following referrals from peripheral facilities with either vaginal or cervical abnormalities. This facility-based recruitment, coupled with structural inefficiency in some rural settings that conveyed referrals, limits the generalisation of the study findings.

CONCLUSION

This study evaluated the diagnostic efficacy of p16/ki-67 cytology as a possible alternative triage for HPV-positive women. Immunostaining showed increased sensitivity in detecting high-grade CIN 2 and above (≥CIN2), although specificity appeared lower compared to VIA. Accordingly, these results suggest that p16/ki-67 cytology is superior to VIA in identifying high-grade CIN and represents a promising approach as an efficient co-test strategy for high-risk HPV-positive women.

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