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ENHANCING CERVICAL CANCER TRIAGE: PROSPECTIVE EVALUATION OF P16/KI67 DUAL STAINING IN VIA-POSITIVE PATIENTS

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ABSTRACT Cervical cancer remains a significant cause of cancer-related mortality among women, particularly in developing countries. This study aimed to evaluate the potential of p16/Ki67 dual staining as a method for enhancing cervical cancer triage in VIA-positive patients.This prospective study was conducted at a tertiary care hospital over 18 months. Premenopausal women aged 30-50 years were opportunistically screened for cervical cancer using Visual Inspection with Acetic Acid (VIA). VIA-positive patients were subjected to colposcopy and guided biopsy. p16/Ki67 dual staining was performed on alcohol-fixed cervical smears using antibodies against p16 and Ki67 antigens. Data was analyzed using SPSS version 22, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of p16, Ki67, and combined p16/Ki67 were calculated using histopathology as a gold standard.Out of 700 screened women, 70 were VIA-positive. Histopathological examination revealed various cervical lesions, including benign findings (47%), Cervical Intraepithelial Neoplasia (CIN) grade 1 (25.7%), CIN 2 (15.7%), CIN 3 (7.1%), and squamous cell carcinoma (4.3%). VIA demonstrated moderate diagnostic accuracy and PPV for detecting ≥CIN1 and ≥CIN2 lesions. Liquid-Based Cytology exhibited higher sensitivity for detecting cervical lesions. The combined evaluation of p16ink4a and Ki67 expression revealed promising insights into cellular changes associated with VIA-positive cases.The study underscores the potential of p16/Ki67 dual staining as a valuable tool for enhancing cervical cancer triage. VIA remains a cost-effective screening method, and the introduction of p16/Ki67 immunostaining holds promise for accurate triaging of VIA-positive patients. This approach can potentially reduce overtreatment and improve healthcare cost-efficiency. Further research is needed to refine the diagnostic accuracy and optimize the management of cervical lesions, and broader adoption of p16/Ki67 immunostaining is recommended for triaging VIA-positive women.

KEYWORDS : CIN, Dual staining,Visual Inspection Acetic acid, Triage test, Liquid Based Cytology, p16/Ki67,Cervical cancer screening

INTRODUCTION

CIN, Dual staining,Visual Inspection Acetic acid, Triage test, Liquid Based Cytology, p16/Ki67,Cervical cancer screening.[1]

The progression of precancerous cervical lesions ie cervical intraepithelial neoplasia (CIN) into invasive cervical cancer typically occurs over a time of 10 to 15 years following persistent infection with Human Papillomavirus (HPV). It is a double stranded DNA virus. The disease burden of age adjusted prevalence of CIN2 and CIN3 among Indian women in some studies is as high as 0.5% and 0.4% respectively.[2]This period of about 10 years presents numerous opportunities for the timely detection and treatment of these lesions. Despite being asymptomatic, these lesions can be identified effectively through screening methods. Early detection offers a good chance of complete cure through simple procedures, whereas less than one-third survival rates are observed for advanced stages of cervical cancer. Unfortunately, population-based screening services are scarce in our setting due to logistical challenges. Therefore alternative options are under exploration.

Cervical cytology has resulted in decrease in cervical cancer incidence in developed countries. In conventional cytology exfoliative cells of cervix are smeared on glass slide and fixed by 95% ethanol. Liquid based cytology (LBC) is a newer method and is superior in sensitivity and specificity. Sure-Path and Thin-prep are two regularly utilized preparations. Sure-path media are largely ethanol-based, while thin-prep media are methanol-based. LBC samples have the advantage to be used for other tests as well like for HPV, chlamydia or gonorrhoea.The disadvantage with cytology other than the requirement of consumables are need for laboratories , trained pathologists , more time for interpretation , more than one visit ,screening programs etc which translates into financial burden.

Visual Inspection with Acetic Acid (VIA) is an affordable and simple screening technique employed for the detection of cervical abnormalities, especially in low resource settings. During VIA, acetic acid is applied to the cervix, inducing protein denaturation in the epithelial cells which is responsible for the white color. VIA is a valuable screening tool, though it still has intrinsic limitations. The accurate interpretation relies on the experience and expertise of the healthcare provider, and there is a possibility of both false-positive and false-negative results.[3]

Worldwide 71% of cervical cancer is associated with HPV 16-18.[4] The HPV genome encodes six early regulatory proteins (E1, E2, E4, E5, E6, and E7) and two late structural proteins (L1 and L2). E6 and E7 genes encode for oncoproteins that play a key role in carcinogenesis. E6 binds to p53 and causes its degradation, while E7 binds to Rb. p16^{ink4a} is a tumor suppressor protein that acts as a cyclin-dependent kinase inhibitor. It plays a pivotal role in regulating the cell cycle by inhibiting the progression of cells into the S phase. One of its key functions is facilitating the reformation of the Rb protein and E2F transcription factor complex. [5] Specifically, the E7 oncoprotein interferes with the Rb/E2F pathway, causing overexpression of p16 ^{ink4a} by a negative feedback in HPV-transformed cervical epithelial cells. [6]

Ki67 is regarded as a marker for cellular proliferation and is a nuclear protein. In a cell infected by high risk (HR) HPV the coexistence of p16 and Ki67 within the same cell signifies the dysregulation of the cell cycle. When both p16 and Ki67 demonstrate positive expression, it is regarded as dual stain positivity. This provides valuable insights into the proliferation dynamics of cells affected by HR HPV.[7]

This present study was undertaken to find the sensitivity and specificity p16^{ink4a} /Ki67 dual staining for detection of high grade cervical lesions in patients with VIA positivity.

METHODS

This prospective cross sectional study was carried out in the Department of Obstetrics and Gynecology, in a tertiary care hospital over18 months. The sample size for our study was based on a study by Haripriya Vedantham et al.[8] who reported that prevalence of VIA positive was 12.74%.The calculated sample size with 95% confidence interval (CI) and 5% margin of error was 136 but we could not recruit that many patients as it was a hospital based time bound study.

All procedures performed in this study involving human participants

were per the ethical standards of the institutional ethics committee and with the 1964 Helsinki Declaration and its later amendments. The study was approved by the Institutional Ethics Committee (IEC) of the institute.

Among premenopausal women aged 30-50 presenting with any gynecological concerns at Outpatient Department (OPD), opportunistic cervical cancer screening using VIA was performed. 5% acetic acid solution was applied on all patients by a cotton swab to the cervix, and the results were assessed after one minute.A clearly visible acetowhite area within the transformation zone was taken as positive. Women who tested positive during the screening and consented for the study were subsequently invited for colposcopy. Prior to colposcopy ,detailed history and comprehensive examination was done, followed by sampling for LBC and $p16^{ink4a}$ ki67 immunostaining. The alcohol fixed cervical smear was stained for dual staining ($p16^{ink4a}$ /ki67) using BIOGENEX and BIOCARE antibodies directed against $p16^{ink4a}$ and ki67 antigen. A positive result was interpreted as brown cytoplasmic staining for $p16^{ink4a}$ expression and red nuclear staining for ki 67 expression.

A total of 700 women were screened ,of which 70 were VIA positive. Subsequently they were subjected to colposcopy directed biopsy. Cervical biopsies were processed in the Pathology Department.

A video colposcope (Digital Colposcope with workstation, Goldway) was used to perform colposcopy. The cervix was examined in good lighting. Saline was used to clear mucus or any vaginal discharge. Any area suggestive of leucoplakia was noted. Green filter was used to look for abnormal vessels. Freshly prepared 5% acetic acid was liberally applied over cervix and vaginal walls. After one minute of application, the entire cervix was examined under magnification (5-25 X). Lugol's iodine solution was then applied so that any abnormal iodine non-uptake areas would stand out as mustard/canary yellow against the mahogany brown colour of the normal squamous epithelium. Swede scoring was done for all patients. Cervical biopsies were taken from abnormal areas. The specimen was fixed in 10% formalin and sent for processing. Biopsy with CIN 2 or worse was taken as positive.

Pregnant women, post hysterectomy patients, known cases of carcinoma of cervix and endometrium, those who have been previously treated for CIN or carcinoma cervix, women with history of pelvic irradiation and women with a frank growth on cervix were excluded from the study.

It was a cross sectional prospective study. Data was analysed using SSPS version 22 .The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of $p16^{ink4a}$, ki67 and combined $p16^{ink4a}$ /ki67 were calculated with histopathology serving as a gold standard.

RESULTS

Out of 700 screened women, 70 tested positive on VIA ie 10%.The study population ranged between 30 and 50 years of age, with a mean age of 42. years. Majority of women reported the age at marriage as 21-25 years. In terms of parity, most (40%) were para 2. The most common presenting symptoms among the VIA-positive women were vaginal discharge and lower abdominal pain.

Histopathology revealed that out of the 70 women in the study, 33 (47%) had benign findings such as squamous metaplasia and chronic cervicitis, 18 (25.7%) had CIN 1, 11 (15.7%) had CIN 2, 5 (7.1%) had CIN 3, and 3 (4.3%) were diagnosed with squamous cell carcinoma. (Figure1)

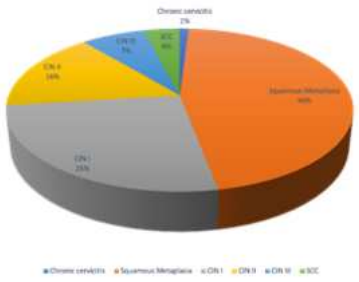

Figure 1: Pie chart depicting distribution of Histopathology in the study population

For the detection of ≥CIN1, VIA showed a diagnostic accuracy of 52.9%, a PPV of 52.9% and a false positivity rate of 47.1%. Comparably, for the detection of ≥CIN2 the diagnostic accuracy of VIA-positive cases was 27.1%, with a PPV of 27.1% and a false positivity rate of 72.9%.

Comparing LBC results with biopsy findings the sensitivity, specificity, PPV, and NPV for detecting. CIN1 or higher lesions were 86.5%, 100%, 100%, and 86.8%, respectively. For detecting CIN2 or higher lesions, the sensitivity, specificity, PPV, and NPV were 100%, 74.5%, 59.4%, and 100%, respectively. The comparative assessment of CIN1 + by various modalities done in the study is shown in Table 1.

Table – 1 Diagnostic Accuracy Of Present Study

Present study (VIA +ve population)	Sensitivity	Specificity	PPV	NPV
For CIN 1+ or more				
LBC	86.5%	100%	100%	92.9%
P16	56.8%	100%	100%	77.1%
KI67	56.8%	100%	100%	77.1%
P16+KI67	59.5%	100%	100%	78.6%
COLPOSCOPY	86.5%	100%	100%	92.9%

Among the total 70 VIA-positive women, 21 exhibited positive expression of $p16^{ink4a}$ and ki67, while 49 were negative for $p16^{ink4a}$ and ki67 ie Dual stain ($p16^{ink4a}$/ki67) positivity rate was 30% for VIA-positive patients.

During colposcopy examination, 28.6% of the VIA-positive cases showed low-grade lesions, 17.1% had high-grade lesions, and 54.3% had normal findings.

$p16^{ink4a}$+ki67 immunostaining results compared with biopsy findings revealed Sensitivity, Specificity, PPV, and NPV for CIN 1 or higher lesions as 59.5%, 100%, 100% and 68.8% respectively. While for CIN 2 or higher lesions as 94.7%, 92.2%, 81.8% and 97.9% respectively as shown in Table 2.

Table -2 Comparison Of Sensitivity, Specificity, Ppv And Npv Of P16+ki67 For Cin1+ And Cin2+

P16+ki67 immunostaining	CIN1+ or more	CIN2+ or more
Sensitivity	59.5% (42-75)	94.7% (74-100)
Specificity	100.0% (89-100)	92.2% (81-98)
PPV	100.0% (85-100)	81.8% (60-95)
NPV	68.8% (54-81)	97.9% (89-100)

We found that $p16^{ink4a}$ & ki67 in LBC cell block is an accurate test for detecting pre-invasive lesions.

DISCUSSION

Cervical cancer has been declared as one of the very few preventable diseases by the World health Organisation (WHO). Implementation of cervical cancer screening programs have been shown to decrease the incidence of invasive cervical cancer and cancer related deaths.[9]

Precancerous lesions have the potential to develop into cervical cancer if left untreated. Most patients exhibit detectable dysplasia approximately 8-10 years prior to the development of invasive malignancy.[10]

In low-resource settings, VIA is a commonly used screening method, with the added benefit of low cost and performance by paramedical staff in outpatient clinics and camps. The reported VIA positivity has been documented to vary between 6.6% and 27.4%. [11]Our observed rate of 10% is consistent with this range.

A study conducted by R. Sankaranarayanan et al demonstrated that despite its limitations, utilization of VIA is a practical strategy in limited resource settings, like ours, a quality-assured single visual inspection with acetic acid exhibits a realistic sensitivity of approximately 50%.[12]

In a meta-analysis, including 29 VIA and 19 visual inspection with Lugol's iodine (VILI) studies, by Liang Qiao et al it was found that VIA had a sensitivity of 73.2% and specificity of 86.7% for CIN2+.VIA and VILI showed greater sensitivity for more severe outcomes, suggesting VIA, VILI, or their combination could be effective options for cervical screening in low-resource settings. [13]

Thus, according to various studies sensitivity of VIA ranges between 80- 89% and specificity between 87%-92%. [8,12] VIA Positive patients, should be triaged using Colposcopy and guided biopsy, ie the screen, triage and treat pathway, as the "Screen and Treat" method has been associated to overtreatment.

Consequently, in our resource-constrained settings, utilizing HPV triage for VIA-positive women is not a highly practical choice due to its cost, limited availability, lack of specificity, and inability to identify histological transformation. Healthcare workers at even primary health centers can collect smears for p16^{ink4a} and ki67 immunostaining from VIA-positive individuals, which can then be sent to a centralized laboratory. Interpretation of p16^{ink4a} and ki67 slides can be done by trained workers as shown by Wentzensen et al. They found high interobserver concurrence among a trained cytologist and one with nominal training in cytology.In their study, they also found that dual staining served as an useful method of risk stratification in all HPV positive women, including those with normal cytology. [14] The presence of p16^{ink4a} and ki67 positivity would also help identify VIA-positive women with CIN, enabling the accurate identification of those who require immediate treatment. Therefore, p16 ^{ink4a} and ki67 immunostaining hold potential in VIA based screening programs.
In 2005, N Murphy and colleagues researched the use of p16 ^{ink4a} predictive indicators in preinvasive neoplasia of the squamous and glandular types. A largely significant linear relationship between p16 ^{ink4a} and increasing degrees of squamous dysplasia was discovered using basic linear regression analysis.[15]

In a 2020 study by Wang et al. the effectiveness of p16^{ink4a} as a diagnostic marker was investigated for CIN2+. According to their study, immunohistochemistry staining of p16 ^{ink4a} was identified as a reliable and effective diagnostic means for distinguishing between samples with and without CIN2+ cells.[16]

Studies on the diagnostic performance of p16 ^{ink4a} +ki67 by different authors in comparison to the current study showed similar findings in terms of sensitivity, specificity and negative predictive value as shown in Table 3.

Table-3 Diagnostic Performance Of Combined P16+ki67

Study	Year	Population	Sensitivity	Specificity	PPV	NPV
Present study for CIN1+	2021	70 VIA positive	59.5%	100%	100%	68.8 %
Present study for CIN2+	2021	70 VIA positive	94.7%	92.2%	81.8 %	97.9 %
Chadha S et al for CIN2 +[17]	2023	89 (ASCUS +LSIL)	66.7%	84.8%	25%	97.1 %
Magkana M et al[18] For CIN2+	2020	200 (ASCUS+LSIL)	90.4%	97.2%	90.4 %	97.2 %
Ren C et al[19] For CIN2+	2019	300 (ASCUS)	50%	75.2%	11.11 %	96.05 %
Zhu Y et al[20] For CIN2+	2019	300 (ACSUS)	98.2%	82.5%	55.2 %	99.5 %
Liao GD et al[21] For CIN2+	2018	198 (ASCUS + HR HPV)	94.1%	80.1%	30.8 %	99.3 %

Chadha S et al concluded that p16^{ink4a} ki67 Dual staining was a valuable triage method for low grade cytology ie Atypical squamous cells of undetermined significance (ASCUS) and Low-grade squamous intraepithelial lesion (LSIL) with higher specificity and accuracy than HPV 16/18 genotyping. [17]

Magkana M et al studied the utility of p16 ^{ink4a} /ki-67 in Greek population with ASCUS and LSIL. They inferred that p16^{ink4a} /ki-67 is a safe and rapid assay that could be used to detect CIN2+ among women with mild cervical lesions, presenting both high-level sensitivity and specificity. [18]

Ren C et al. conducted a study suggesting that p16 ^{ink4a} /ki-67 immunocytochemistry was an important tool for ASCUS triage, although it may lead to a trade-off between improved diagnostic specificity and reduced sensitivity compared to HPV genotyping. [19]

Thus dual staining exhibits substantial promise as a triage modality subsequent to any primary screening assessment, including VIA, cytology, or HPV testing, demonstrating robust sensitivity and specificity in the identification of high-grade lesions, as supported by multiple studies.

The current study showed a similar result while applying the dual stain triage method to VIA positive patients. The application of this novel idea of performing p16^{ink4a} and ki67 in LBC cell block, applicability to VIA positive patients, prospective design and use of histopathology as gold standard were among the strengths of the current study.The small sample size and highly selected population of this study were the limiting factors.

CONCLUSIONS

In conclusion, our study confirms the moderate diagnostic accuracy and positive predictive value of VIA in detecting CIN 1,2 (≥CIN1) and or higher (≥CIN2) lesions. LBC demonstrates higher sensitivity but lower specificity and PPV for identifying cervical lesions. Additionally, the combined assessment of p16ink4a and ki67 expression offers indispensable insights into the cellular changes associated with VIA-positive cases.

While our findings suggest the potential to reduce rates of overtreatment and healthcare costs through the application of p16ink4a/ki67 dual staining, it is essential to note that a formal cost-effectiveness analysis was not conducted in this study. Further research is warranted to explore the economic implications and optimize the management of cervical lesions.

Our study underscores the need for ongoing efforts to improve diagnostic accuracy in cervical cancer screening programs. The ability of p16ink4a immunostaining to distinguish between transient and persistent HPV infections holds promise in overcoming the limitations of VIA. However, challenges related to cost and limited availability of p16ink4a and ki67 immunostaining remain significant barriers to be addressed in future research.

Based on our findings, we recommend the consideration of immunostaining with p16ink4a and ki67 on a larger scale for triaging VIA-positive women, potentially enhancing the effectiveness of cervical cancer screening programs. Future studies should focus on refining this approach and addressing the identified limitations to advance the early detection and management of cervical lesions.

Conflict of Interest: None.

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