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ORIGINAL ARTICLE

Evaluation of Biomarkers p16/Ki-67 in Cervical Cytology for Diagnosis of Cervical Intraepithelial Neoplasia

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Abstract

Purpose To test the clinical utility of biomarkers p16/Ki-67 expression in cervical cytology smears as a marker for transforming HPV infection.

Setting and Design Experimental study at a tertiary care hospital.

Methods Women who were screened positive on Pap and visual inspection tests (n = 280) underwent colposcopy and biopsy. p16/Ki-67 immunostaining was performed in abnormal Pap smears (n = 86), and HPV DNA testing was also performed in the same women.

Statistical Analysis Sensitivity, specificity, negative predictive value, positive predictive value and likelihood ratios were calculated for each biomarker separately and in combination. McNemar test and receiver operating characteristic (ROC) curves were used to compare sensitivity and specificity of biomarkers with HPV DNA. Areas under the ROC curve (AUC) were compared using the Chi-square test.

Results Eighty-six women with abnormal cytology were evaluated with p16/Ki-67 immunocytochemistry; 20.9% (n = 18) and 18.6% (n = 16) were positive for each biomarker, while dual marker was positive in 15% (n = 13). In all smears, the sensitivity of $p16^{INK4a}$/Ki-67 in detecting CIN 2+ lesion was 76.9% and specificity was 95.8%. For ASCUS (n = 42) and LSIL (n = 23) smears, specificity and negative predictive value of p16/Ki-67 for CIN 2+ were 100% with a likelihood ratio (LR+) of 27 and 25, respectively, suggesting good diagnostic accuracy. In comparison with HPV DNA testing, combined marker p16/Ki-67 was significantly more specific (p = 0.003); AUC was 0.734 and 0.635, respectively.

Conclusions p16/Ki-67 evaluation in cervical cytology is a valuable biomarker in triaging for CIN 2+ disease in ASCUS and LSIL smears.

Keywords p16/Ki-67 · HPV · Cervical cancer screening · Cervical cytology · Biomarker

Introduction

Cervical cancer is the fourth most diagnosed cancer worldwide and the second most common cancer in India. According to GLOBOCAN 2018, 18.1 million new cervical cancer cases were reported worldwide, resulting in 9.6 million deaths. Among them, 96,922 new cases were diagnosed in India, resulting in the death of 60,078 women every year [1]. It remains a major public health problem in developing countries where more than 85% of these cases and deaths occur. Current cervical cancer screening tests including Papanicolaou (Pap) test, visual screening methods and human papillomavirus DNA (HPV DNA) testing have a wide variation in sensitivity and specificity in detecting cervical neoplasia. Cytology has a variable

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sensitivity between 47 and 62% and specificity between 60 and 95% for high-grade cervical intraepithelial neoplasia (CIN 2+) lesions [2]. Visual inspection with acetic acid (VIA) has a high sensitivity of 71.4–90%, but has a low specificity of 36–50%, leading to overtreatment [3, 4]. Although HPV DNA testing has high sensitivity and specificity of 97% and 93%, respectively, it fails to diagnose persistent infections, leading to increased colposcopy referrals and overtreatment [5].

To optimize the accuracy of Pap-based screening and reduce overdiagnosis by HPV DNA testing and visual screening, various cellular markers have been identified to detect transforming or persistent HPV infection. These disease-specific biomarkers include tumor suppressor protein $p16^{INK4a}$ (p16) and Ki-67 that is expressed in proliferating cells. The simultaneous expression of both can be used as a surrogate marker of cell cycle deregulation mediated by transforming HPV infection [6].

The studies on dual staining have shown high sensitivity (92–94%) and specificity (80.6–68%) in identification of CIN 2+ lesions in women with atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesion (LSIL) via cytology, respectively [7]. However, a systematic review of five studies by Kisser and Zechmeister-Koss concluded that further studies on test performance of p16/Ki-67-based triage for women with ASCUS or LSIL cytology are needed and routine testing cannot be recommended due to insufficient high-quality evidence [8].

The aim of the present study was to test the clinical utility of biomarkers p16/Ki-67 expression in cervical cytology as a marker for transforming HPV infection and progression to cervical intraepithelial neoplasia (CIN). The primary objective was to study the biomarkers p16/Ki-67 expression by immunostaining in cervical scrapes of screen-positive women and correlate with histopathology. The secondary objective was to compare the accuracy of p16/Ki-67 with HPV DNA testing for identification of CIN 2+ lesions in women with low-grade cervical cytology.

Methods

A clinicopathological study was conducted in Department of Obstetrics and Gynecology and Department of Pathology at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India, between November 2013 and April 2015.

For estimating 90% sensitivity of p16/Ki-67 considering biopsy as the gold standard with 5% precision on either side and 5% level of significance and assuming 50% prevalence of biopsy-positive cases in women who undergo colposcopy, a sample size of 280 women undergoing colposcopy was required. The study was approved by the institutional ethics committee. Informed consent was obtained from all individual participants included in the study.

Sexually active women in the age group of 30–55 years were screened by VIA, visual inspection with Lugol's iodine (VILI) and/or Pap smear. For VIA, acetic acid (5%) was applied using a cotton swab soaked in acetic acid over the exposed cervix and findings were read after 1 min and interpreted as per the International Agency for Research on Cancer (IARC) guidelines [9]. Lugol's iodine was applied on the cervix and yellow-brown stain was interpreted according to IARC charts for VILI.

Postmenopausal women, women with an obvious cervical growth, acute cervicitis or prior surgery on cervix and pregnant women were excluded from the study.

Screen-positive women on visual inspection and/or Pap smear were included in the study and underwent colposcopy. Cervical scrapes (Pap smear) for p16/Ki-67 and samples for HPV DNA testing were again taken before colposcopy. Colposcopy findings were noted using International Federation of Cervical Pathology and Colposcopy (2011) criteria [10]. Suspicious areas were biopsied using a cervical punch biopsy forceps, and if colposcopy was normal, biopsy was not taken and was considered negative for CIN. History and examination details of all included women were recorded.

All women with abnormal Pap smears (n = 86) underwent p16/Ki-67 immunostaining in cervical scrapes. Out of 86 abnormal Pap smears, atypical squamous cells of undetermined significance (ASCUS) was seen in 42 cases, low-grade squamous intraepithelial lesion (LSIL) in 23 cases, high-grade squamous intraepithelial lesion (HSIL) in 16 cases, atypical squamous cells, cannot exclude HSIL (ASC-H), in two cases, atypical glandular cells (AGUS) in one case and squamous cell cancer in two cases. Primary antibody used for p16 was Bio SB Mouse Monoclonal antibody (clone: 16P04, JC2) and for Ki-67 was Rabbit Monoclonal Antibody (SP6). They were poured on the sections taking care of even distribution followed by application of secondary (biotinylated) antibody and tertiary antibody and then counterstained with hematoxylin, washed under running tap water and air-dried. The findings of p16 were interpreted by multiplying the intensity of p16 staining with the percentage of positive cells. Ki-67 scoring was done depending on the percentage of cells stained as negative, intermediate and strongly positive. HPV DNA testing was performed by PCR 34 using consensus primers (MY09 and MY11) of the L1 region of HPV genome.

The study flow diagram is shown in Fig. 1.

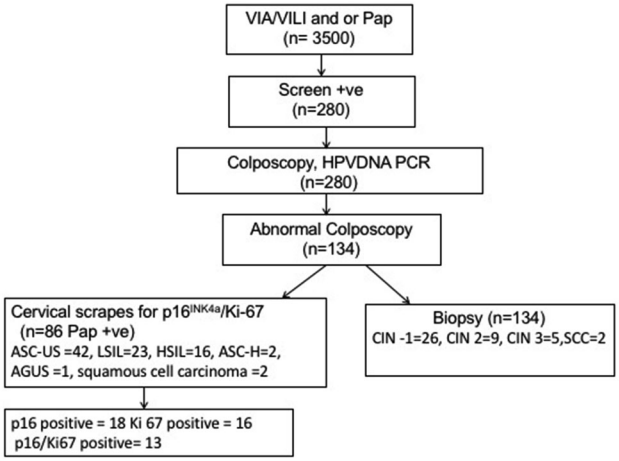

Fig. 1 Study flow diagram

Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR−) of the test were calculated, taking biopsy as the gold standard. Normal biopsy/colposcopy findings were taken as negative. McNemar test was used to compare sensitivity and specificity of biomarkers with HPV DNA and histopathology. Performance of biomarkers and HPV DNA test for predicting high-grade cervical intraepithelial neoplasia (CIN 2+) was evaluated by the receiver operating characteristic (ROC) curve. Areas under the ROC curve (AUC) were compared using the Chi-square test. A value of p less than 0.05 was considered statistically significant.

Results

Women who were screened (n = 3500) and tested positive (n = 280) underwent colposcopy and HPV DNA testing. Out of all 280 screen positives, 231 were VIA positive, 86 had abnormal cytology, while 49 were HPV DNA positive. Immunocytochemistry with p16 and Ki-67 was done in 86 women with abnormal Pap test results. The baseline variables are summarized in Table 1.

Colposcopy was performed in 280 screen-positive women out of whom 51.4% (n = 146) had normal findings, while 33.2% (n = 93) and 14% (n = 39) had minor or major lesions and two cases were suspicious for invasion. 85.7% (n = 265) women had a transformation zone (TZ) 1. The rest of the 15 (5.3%) women who had TZ 2 and 3 underwent endocervicoscopy. Punch biopsy was taken in 134 cases out of whom 31.3% (42) women had abnormal histopathology: 19% (n = 26) cervical intraepithelial neoplasia (CIN)-1, 6.6% (n = 9) CIN 2, 3.6% (n = 5) CIN-3 and two squamous cell carcinoma.

Table 1 Baseline characteristics

Variable	n = 280
Age (years)*	38.70 (± 7.795)
Age of first sexual intercourse (years)*	18.64 (± 2.576)
Age of marriage (years)*	18.66 (± 2.576)
Age of first child birth (years)*	20.47 (± 2.92)
Parity (median)	3
Socio economic status	Lower (84.6%)
Religion	Hindu (77.8%)
Multiple sexual partners	(4%)
Mean age of menarche	13.85 (SD ± 0.76)
Menstrual complaints	
Irregular cycles	10 (3.57%)
Intermenstrual bleeding	37 (13.2%)
Post-coital bleeding	27 (9.6%)
Other symptoms	
Vaginal discharge	117 (41.78%)
Pain abdomen	37 (13.21%)
Low back ache	6 (2.14%)
Dyspareunia	2 (0.7%)
Contraceptive usage	110 (39%)
Ligation	50 (45.45%)
Copper T	30 (27.27%)
OCP	17 (15.45%)
Barrier	13 (11.8%)
Smoking	14 (5%)

*reflects mean +/− SD

Out of 65 low-grade smears (ASCUS and LSIL), CIN-1 and CIN 2 were reported in 16.9% (n = 11) and 6% (n = 4) cases, respectively. Out of 16 cases of HSIL, CIN-1 and CIN 2 were reported in 12.5% (n = 2) each and CIN-3 in 31.2% (n = 5).

HPV DNA testing was positive in 15.7% (n = 48) out of 280 women. The sensitivity of HPV DNA PCR test was 40.48%, specificity was 86.9%, PPV was 35.4%, NPV was 89.2%, LR+ was 3.11 and LR− was 0.68. The detection rate of HPV DNA PCR test in CIN-1 was 26.9% and in CIN 2+ lesion was 62.5%.

Out of 86 positive smears, p16 was positive in 18 (20.9%) cases, Ki-67 in 16 (18.6%) and combined staining in 13 (15%) (Figs. 2, 3). The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of p16 in detecting CIN 2+ lesion were 84.6, 90.4, 97 and 61%, respectively. The sensitivity of Ki-67 in detecting CIN 2+ was 84.6%, specificity 93.1%, PPV 88.7% and NPV 97%. For dual staining (p16/Ki-67), sensitivity, specificity, PPV and NPV were 76.9, 96, 77 and

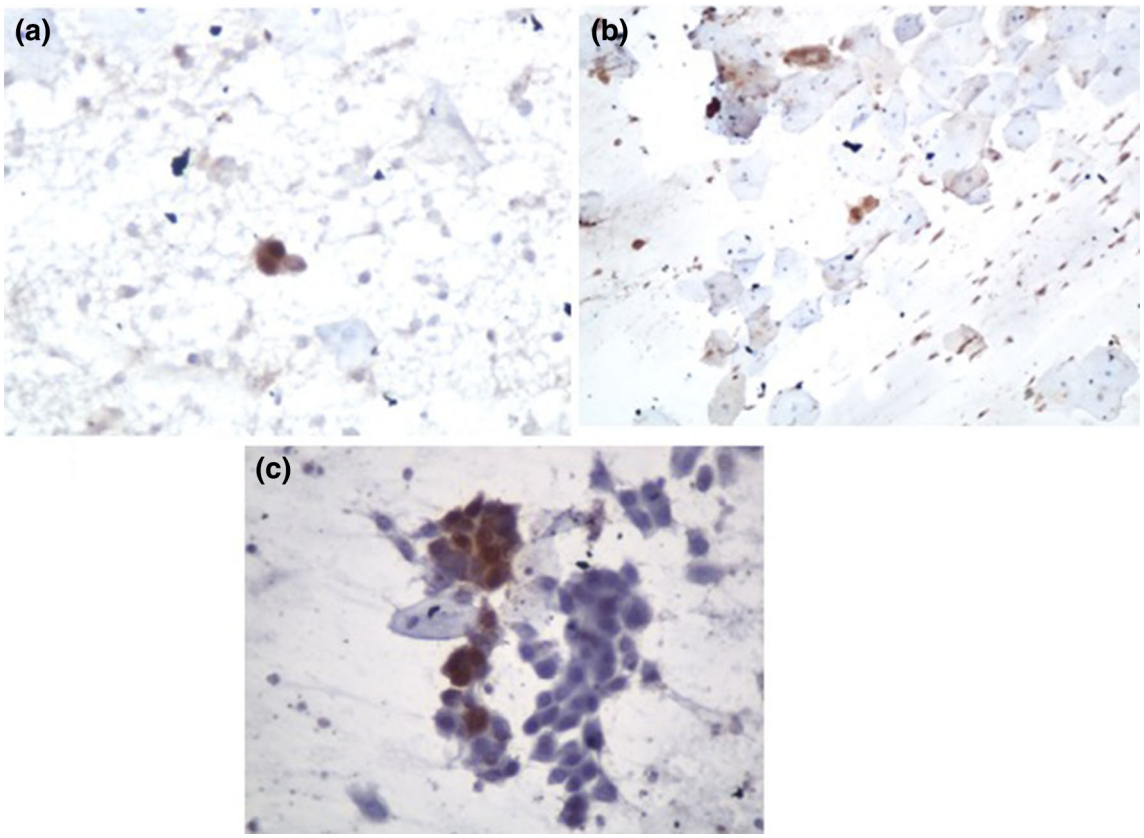

Fig. 2 p16 staining in Pap smears

95.89%, respectively. For combined staining, the positive and negative likelihood ratios were 18.72 and 0.24, respectively.

The performance of biomarkers and HPV DNA testing for diagnosis of CIN 2+ disease in low-grade smears (ASCUS and LSIL) is shown in Table 2. The specificity and positive likelihood ratio of p16/Ki-67 were significantly more than those of HPV DNA testing for diagnosis of CIN 2+ lesions. Combined marker p16/Ki-67 was significantly more accurate than HPV DNA ($p = 0.003$); area under the curve was 0.734 and 0.635, respectively (Fig. 4).

Discussion

Immunocytochemistry for p16/Ki-67 was performed in cervical cytology smears of 86 women out of 280 women who had undergone colposcopy. The specificity of p16, Ki-67 and dual stain (p16/Ki-67) for diagnosis of CIN 2+ disease was high (90.4–96%). The sensitivity of p16/Ki-67 was similar to that of HPV DNA testing (50%), but specificity was significantly higher (96.7% vs 77%; $p = 0.03$) for diagnosis of CIN 2+ disease in low-grade smears.

The incidence of CIN 2+ disease was 6% in ASCUS and LSIL cytology in the present study. The results are similar to other studies which report an incidence of 7–8% of CIN 2/3 in ASCUS smears [11, 12]. In LSIL smears, the diagnosis of precursor lesions (CIN 2/3) has been variably reported between 9 and 30% [13].

In the present study, p16 was positive in 62% HSIL compared with 7–13% in low-grade smears. Bibbo et al. in his study also showed a higher positivity of p16 in HSIL as compared to LSIL smears (96% vs 74%) [14].

For p16 immunostaining, Gustinucci et al. showed sensitivity and specificity of 91% and 64% for CIN 2+, respectively, in ASCUS smears ($n = 213$) and 77% and 64% , respectively, in LSIL smears ($n = 186$) [15]. However, in the present study the sensitivity was low and specificity was high for CIN 2+ disease: 50% and 93% for p16 and 75% and 97% for Ki-67, respectively, in low-grade smears (ASCUS and LSIL). This can be explained due to different sample sizes in the two studies. However, both studies show a high negative predictive value (96–100%) of p16 for CIN 2+ in low- and high-grade smears.

While correlating the immunostaining with histopathological results, in the present study out of 86 smears, p16 was negative in 11 cases of CIN-1 and two cases of CIN 2, while all cases of CIN 3 were p16 positive. Hence, the majority of the lesions of CIN-1 which were p-16 negative

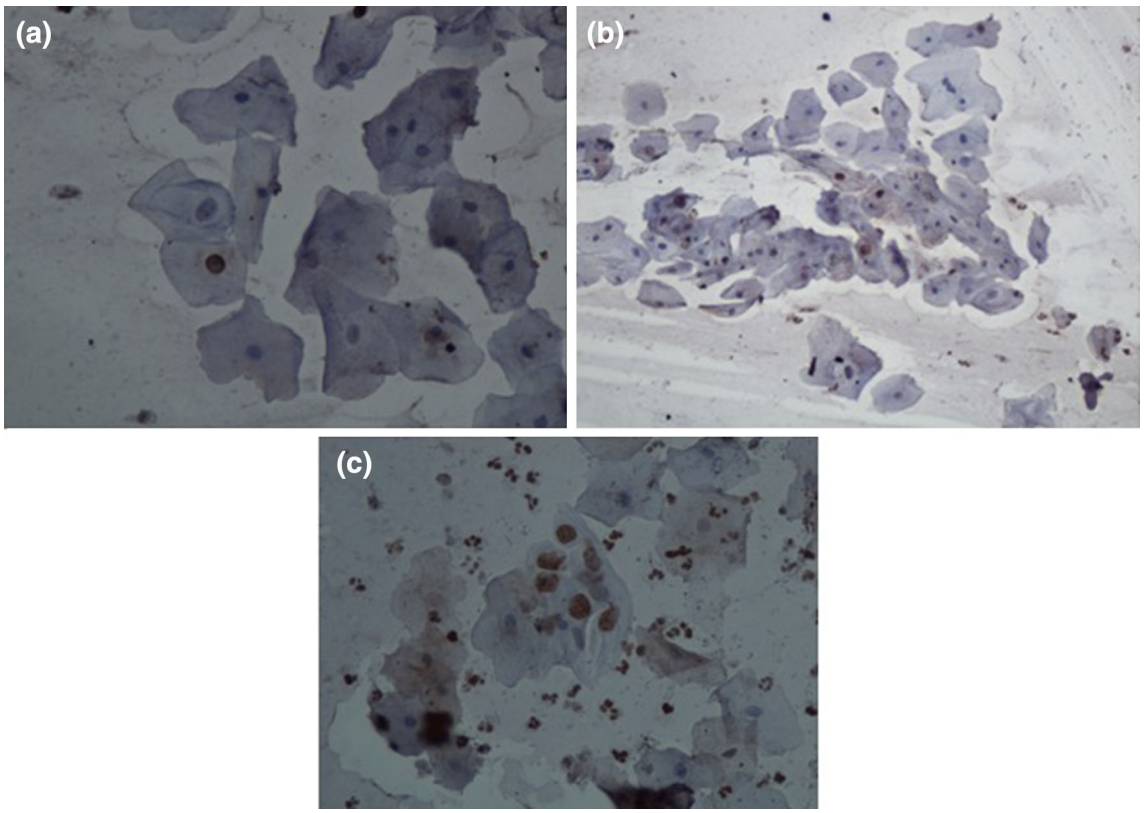

Fig. 3 Ki-67 staining in Pap smears

Table 2 Performance of p16/Ki-67 and HPV DNA in low-grade cytology taking CIN 2+ as cutoff

CIN 2+	p16	Ki-67	p16/Ki-67	HPV DNA
Sensitivity (CI)	50% (6.76–93.2%)	75% (19.4–99%)	50% (6.76–93.2%)	50% (6.7–93.2%)
Specificity (CI)	93.44% (84–98%)	96.7% (88.6–99%)	96.72% (88–99.6%)	77.05% (64–86.8%)
LR+	7.63 (1.95–29.8)	22.87 (5.23–100)	15.25 (2.85–81.72)	2.18 (0.74–6.43)
LR−	0.54 (0.2–1.43)	0.26 (0.05–1.41)	0.5 (0.19–1.38)	0.65 (0.24–1.75)
Positive predictive value	100%	75%	75%	49%
Negative predictive value	82.5%	82.5%	79.6%	89.2%

CI confidence interval, *LR+* positive likelihood ratio, *LR−* negative likelihood ratio

probably indicate non-transforming infection with HPV and thus a self-limiting disease. This, however, needs to be confirmed by well-designed prospective studies. Wang R et al. showed that significantly high number of p16-positive cases of CIN-1 progressed as compared to p16-negative cases (27% vs 7%) and p16 protein staining had a high negative predictive value of 93% for progression to CIN 2–3 [16].

In the present study, since commercially available p16/Ki-67 dual stain was not used, both stains were used separately on the same smear. This was probably the reason for a lower overall positivity of 15% compared with 59% by Wentzensen et al. [17]. The latter also reported a higher sensitivity (86% vs 77% in the present study), but a much lower specificity (60% vs 95.8%), to detect CIN 2+ in all smears. This difference can be explained by the difference in overall positivity and difference in the techniques of staining.

The sensitivity and specificity for p16/Ki-67 have been variably reported between 64% and 98% and 43% and 81%, respectively, across several studies for diagnosis of CIN 2+ disease in low-grade smears, and this difference is due to variation in sample size and interpretation of results [7, 8, 17]. In the present study, the lower sensitivity of 50%

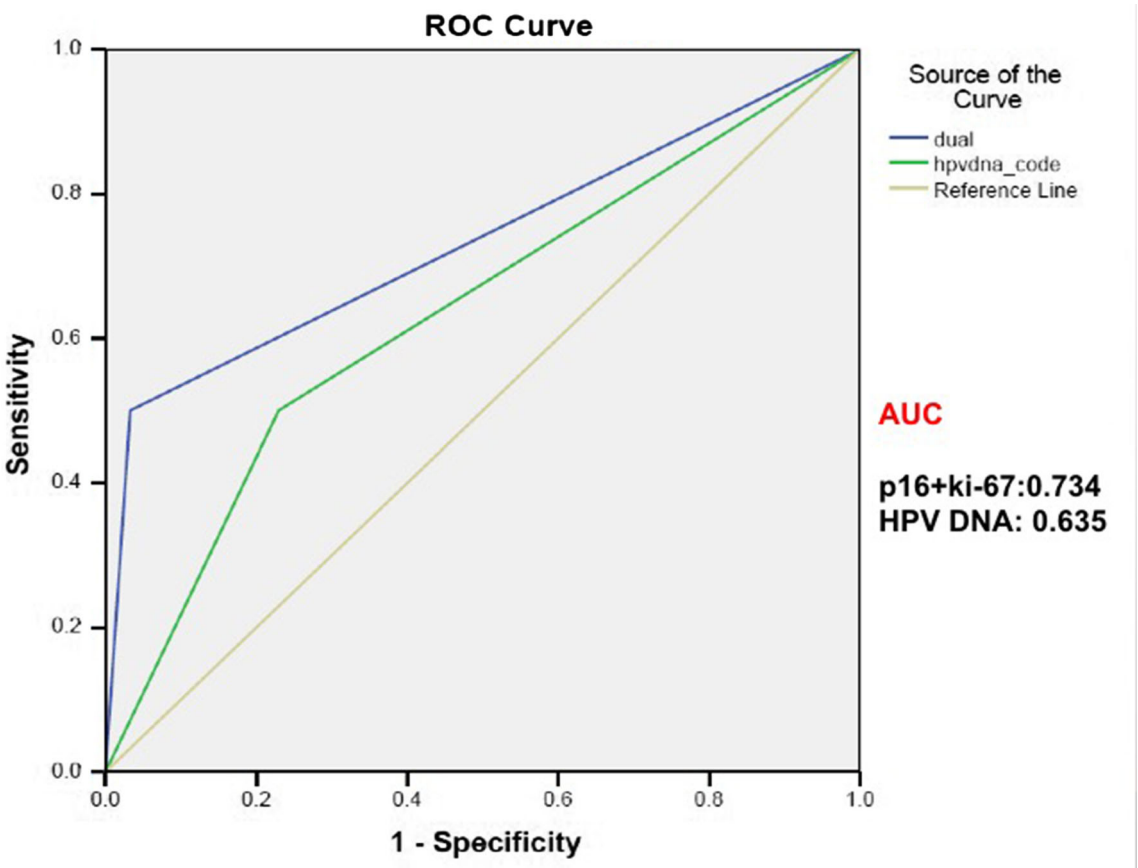

Fig. 4 Receiver operating characteristic curve comparing p16/Ki-67 and HPV DNA testing for triage of low-grade smears

for combined stain is similar to results of Possati-Resende et al. and Edgerton et al. who have also reported a sensitivity of 60% [12, 18]. As shown in other studies, the specificity of combined p16/Ki-67 immunostaining was significantly higher ($p = 0.003$) compared with that of HPV DNA testing for detection of CIN 2+ disease in low-grade smears in the present study [8, 12, 19]. Despite differences in the specificity of dual stain and HPV DNA testing, both tests had high negative predictive value and negative likelihood ratios, indicating that both can serve as effective triage tools.

To conclude, p16/Ki-67 alone or in combination can be used as effective triage tools to predict and identify high-grade precursor lesions, especially in low-grade cytology smears. In comparison with HPV DNA testing, p16/Ki-67 combined staining had greater accuracy in ruling out or detecting CIN 2+ in ASCUS and LSIL smears, thereby avoiding unnecessary interventions and referral to colposcopy.

Compliance with Ethical Standards

Conflict of interest There is no conflict of interest among the authors.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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