Diagnostic value of cell block technique combined with p16/Ki-67 double staining in cervical cancer screening ≥CIN2 with cytological results of ASC-US/LSIL

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Research Article

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Abstract

Background

Accurate screening for ≥ CIN2 in patients with cytologic results of ASC-US/LSIL is key to cervical cancer screening, which will benefit women by reducing the psychosocial impact of having repeated abnormal smears and undergoing colposcopy.

Methods

To analyze the potential role of the p16/Ki-67 double-stained(DS) on cell block (CB) assay on cytological results of atypical squamous cells of undetermined significance(ASC-US) and low-grade squamous intraepithelial lesion (LSIL) in a final pathological diagnosis of ≥ cervical intraepithelial neoplasia (CIN)2 lesion. We screened ASC-US/LSIL cases with complete results of high-risk human papillomavirus (HR-HPV) and DNA ploidy analysis, and based on the principle of informed consent and voluntariness, the patients' remaining Liquid Based Cytology(LBC) specimens were subjected to CB. A total of 222 patients underwent colposcopic biopsy to compare and analyze the diagnostic efficacy of the three screening methods for ≥ CIN2.

Results

The Area Under the Curve (AUC) of p16/Ki-67 DS was significantly higher than that of HR-HPV and DNA ploidy, $P < 0.01$. Net reclassification improvement (NRI) index results showed that compared with HR-HPV and DNA ploidy, p16/Ki-67 DS on CB increased the percentage of patients correctly classified by 147.0% and 127.8%, respectively, $P < 0.01$. The results of the integrated discrimination improvement index (IDI) index showed that p16/Ki-67 increased diagnostic ability by 60.9% and 63.3%, $P < 0.01$.

Conclusion

The p16/Ki-67 DS on CB significantly improved the correct classification of patients with ≥ CIN2, was a good method to triage ASC-US/LSIL patients, and effectively avoided the occurrence of overdiagnosis and treatment.

Introduction

Globally, cervical cancer is the fourth most common malignancy in women, causing about 275,000 deaths each year[1]. Cervical cancer is the result of a long-term process, which is the result of a pre-invasive cervical lesion, i.e. CIN[2]. According to the degree of lesion, there are low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). The development of cervical cancer requires numerous stages, and it is of great clinical significance to detect cervical

precancerous lesions in a timely and accurate manner, predict their development and select appropriate treatment strategies. Detection of HSIL and lesions that may develop into HSIL is the purpose of early cervical cancer screening[3]. Accurate detection and prediction of high-grade intraepithelial cervical lesions (≥ CIN2) are particularly important. ASC-US, first introduced by The Bethesda System (TBS) classification[4] is the most common abnormal cervical cytology finding. ASC-US accounts for approximately 3–5% of cytologic findings on cervical screening in asymptomatic women and accounts for the majority of abnormal cytologic findings[5, 6]. Patients with ASC-US exhibit a wide range of histologic findings, with the majority of patients exhibiting mild abnormalities below CIN2 with spontaneous regression; however, invasive carcinoma is also rare in clinical practice[7]. leading to confusion among clinicians and patients[8].LSIL is among those with mildly abnormal cytologic presentation, and a large percentage of women both do not and may develop high-grade CIN (HGCIN) over time. Minor abnormalities, LSIL and ASC-US, represent a large burden at colposcopy. A11 large proportion of these will not lead to a diagnosis of CIN2 + or CIN3 yet still remain under extensive follow up[9]. Good sensitivity and specificity of p16/Ki-67 double immunostaining (p16/Ki-67 DS) for the detection of cervical high-grade intraepithelial neoplasia (CIN2+) [10–14]. The p16/Ki-67 DS compensates for the shortcomings of a single staining technique by enabling the simultaneous display of two antigens on the same section. In this paper, We have assembled 222 cases of women on whom liquid-based cytomorphologic cervical cancer screening has been performed concurrent with histologic biopsy, DNA ploidy analysis, HR HPV testing, and dual p16/Ki-67 immunohistochemical staining, the latter of which has been performed on cytologic CB sections. To explore the value of P16 / KI-67 DS on CB in triage of ASC-US/LSIL patients.

Materials And Methods

Subjects

Patients who attended the gynecology department of Haidian Maternal and Child Healthcare Hospital in Beijing, China, and underwent colposcopy with positive test results for HR-HPV (HPV16/18/31/33/35/39/45/51/52/56/58/59/ 66/68/82).Data were selected for investigation, and LBC specimens were collected for CB production.

Inclusion and exclusion criteria

Inclusion criteria: women aged 20–79 years; signed patient informed consent for the collection of cervical epithelial exfoliated cells and cervical tissue for this study. A total of 222 ASC-US/LSIL patients with complete results of HR-HPV and DNA ploidy analysis and simultaneous biopsy under colposcopy were selected for the study based on the principle of informed consent and voluntariness. The study was approved by the Ethics Committee of the Haidian District Maternal and Child Health Hospital, Beijing, China, and the review approval number: 2021-2.

Liquid-based cytology

The LBC technique was used for cytological detection. Thin slides were prepared using Thin Prep 2000 and processed to make ultra-thin smears of 13 mm in diameter, then fixed in 95% alcohol, manually Pap stained, sealed and observed microscopically.The experimental consumables and reagents were provided by Guangzhou Anbipin Pharmaceutical Technology Co. Cytology production.

HR-HPV testing

The Cobas HPV detection system (Roche Diagnostic Products, Newjersey, USA) was used. HR-HPV detection and genotyping was performed by polymerase chain reaction (PCR) reaction combined with probes of different fluorescent dyes to detect 14 HR-HPV species (16/18/31/33/35/39/45/51/52/56/58/59/ 66/68) simultaneously. Among them, HPV16/18 results are reported separately.

DNA ploidy analysis

The SPICM-DNA fully automated DNA quantification system and the kits provided by it were used. Each specimen was filmed by liquid-based cell production, stained with Feulgen and all cell nuclei were intergrated optical density (IOD) and nuclear area were measured by the fully automated scanning system. The IOD and DNA index (DI) values were determined by a fully automated scanning system, and the DI value was the ratio of the IOD of the tested cells to the IOD of normal cells.

CB production

The remaining cervical exfoliated cell samples after LBC preparation were poured into 10ml centrifuge tubes (2500r/min) centrifuged for 10 min to enrich the cells, wiped mirror paper placed at the bottom of the embedding box. the supernatant was discarded, the cells at the bottom of the centrifuge tubes were inhaled with a disposable plastic dropper, while melted agar with a concentration of 3% was added to mix and cast into a CB for routine fixation, dehydration, transparent, wax dipping procedure (Sakura tissue dehydrator) to complete the CB.

p16/Ki-67 DS

Immunohistochemical staining was performed with p16/Ki-67 detection kit (purchased from China ZhongShan Jinqiao Company). The CB sections were fixed and antigen-repaired, peroxidase blocking was added, primary antibody incubation, secondary antibody incubation, diaminobenzidine color development, hematoxylin contrast staining and neutral gum sealing were performed, and the sections were interpreted under the microscope (Fig. 1).

Diagnostic criteria

Cytological specimens were reported using the 2001 Bethesda Reporting System standard[4]. Pathological diagnostic criteria were based on the WHO classification criteria for pathology and genetics of breast and female genital tumors(2003): normal or inflammatory response, CIN1, CIN2, CIN3, squamous cell carcinoma and adenocarcinoma. p16/Ki-67 DS on CB diagnostic criteria: p16 was

localized in the cytoplasm and showed brown color; Ki-67 was localized in the nucleus and showed red color. When 1 cytoplasm shows brown color (p16) and the nucleus shows red color (Ki-67), the result is positive for DS, and negative for separate color or no color DS of CBs[15]. According to WHO Classification of Female Genital Oncology (4th edition)[16].

Statistical methods

Excel sheet was applied to enter the data, pathological biopsy diagnosis was used as the final standard, SPSS 23.0 was used to organize and analyze the data, and ROC curves were plotted, and DeLong's test was used for comparison of the area under the ROC curve, DeLong's test, DCA, NRI and IDI were used in the R language pROC, rmda, nricens, and PredictABE packages, respectively, with $\alpha = 0.01$ as the test and $P < 0.01$ as a statistically significant difference.

Results

General information

The 222 ASC-US/LSIL patients ranged from 21 to 74 years, with a median age of 42.5 years. 160 patients (72.1%, 160/222) had positive final histopathological findings, including 58 cases≥CIN2 (26.1%, 58/222), 102 cases CIN1(45.9%, 102/222),62 cases of chronic cervical inflamation(27.9%, 62/222) (Table 1). Among 168 ASC-US cases, 47 biopsies showed ≥CIN2 (30.0%, 47/168). 11 biopsies out of 54 LSIL cases showed ≥CIN2 (20.4%, 11/54). LBC,biopsy pathology, and p16/Ki-67 DS on CB sections were evaluated by two pathologists who were unaware of each other. Reassessment in case of disagreement.

Table 1. Relationship between the results of the 3 screening models and the pathological diagnosis

Sreening models	Pathological Diagnosis		Total
	Positive（≥CIN2）	Negative	
HR-HPV			
Positive	57	142	199
Negative	0	23	23
DNA ploidy			
Positive	49	109	158
Negative	9	55	64
p16/Ki-67 DS			
Positive	50	6	56
Negative	8	158	166

Abbreviations:CIN, Cervical intraepithelial neoplasia;HR-HPV, high risk human papilloma virus;DS,dual-stained.

Evaluation of the effectiveness of screening methods

SPSS23.0 software was used for ROC curve plotting (Figure 2), and DeLong's test was used for the comparison of the area under the ROC curve. The AUC of HR-HPV, DNA ploidy and p16/Ki-67 CB immunohistochemical DS was compared, and the differences were statistically significant. The AUC of P16 / KI-67 DS under ROC curve was 0.900 (P < 0.01), which was significantly higher than HR-HPV and DNA ploidy（Table 2）

Table 2 Comparison of the area under the ROC curve

TEST	AUC	P	DeLong's test[a]	
			Z	P
p16/Ki-67 DS	0.900(0.841,0.958)	<0.001		
HR-HPV	0.557(0.474,0.640)	0.207	11.387	<0.001
DNA	0.583(0.500,0.666)	0.065	7.476	<0.001

Abbreviations:ROC,receiver operator characteristic curve;AUC,areas under the ROC curve;HR-HPV, high risk human papilloma virus.

[a]Comparison with p16/Ki-67.

NRI and IDI index results

It showed (Table3) that p16/Ki-67 increased the proportion of patients correctly classified by 147% and 127.8%, respectively, compared to HR-HPV and DNA ploidy, *P*< 0.01; P16/Ki-67 increased the proportion of ≥CIN2 patients correctly classified by 70.9% and 70.9%, respectively, *P*< 0.01; P16/Ki-67 increased the proportion of ≥CIN2 patients correctly classified by 70.9% and 70.9%, respectively, compared to HR-HPV and DNA ploidy, *P*< 0.01; P16/Ki-67 increased the correct classification of negative patients by 76.0% and 59.6%, respectively, *P*< 0.01. The IDI index results showed that p16/Ki-67 increased diagnostic power by 60.9% and 63.3%, respectively, *P*< 0.01, compared with HR-HPV and DNA ploidy.

TABLE 3 IDI and NRI for different diagnostic indicators

Indicators	p16/Ki67 vs HR-HPV	P	p16/Ki67 vs DNA ploidy	P
IDI (95%CI)	0.609(0.525,0.692)	<0.001	0.633(0.554,0.713)	<0.001
NRI (95%CI)	1.470(1.246,1.470)	<0.001	1.278(1.049,1.278)	<0.001
NRI (+) (95%CI)	0.709(0.500,0.709)	<0.001	0.709(0.510,0.889)	<0.001
NRI (-) (95%CI)	0.760(0.673,0.760)	<0.001	0.569(0.485,0.657)	<0.001

Abbreviations: IDI,integrated discrimination improvement index; NRI,net reclassification

Decision curve (DCA) for different diagnostic indicators (Figure 3)

It can be seen from the figure that the net gain values of p16/Ki-67 are significantly HR-HPV and DNA ploidy in the range of threshold probability of 0.10 to 0.84 interval.

Discussion

HPV as well as LBC testing, has largely reduced the incidence of cervical cancer and morbidity and mortality. Some countries use HPV testing as a primary screening for cervical cancer. The biggest challenge in implementing HPV primary screening is managing the large number of women who are found to have transient HPV infection. HPV testing is considered to have a high sensitivity, but HPV testing can only detect the presence or absence of the virus and cannot distinguish between transient and persistent infections with the virus. The test identifies the virus but not the cancer precursor lesion or the cancer itself[17]. It is well known that the main risk factor for cervical cancer is persistent HPV infection. It promotes impaired cell growth and differentiation, leading to dysplasia (cervical intraepithelial neoplasia, CIN)[18]. Positive HPV test does not mean that cervical lesions have occurred, it indicates an increased risk of future cervical cancer. LBC has low sensitivity and poor reproducibility, and poor LBC staining may lead to misdiagnosis or overdiagnosis. Cytologic findings of ASC-US/LSIL are currently a key area of focus for stratified management of cervical cancer at the screening stage to reduce unnecessary biopsies and avoid overtreatment. Cytology is the test of choice for classifying HPV-positive women because it is highly specific, but it is a subjective judgment and its accuracy depends on the cytologist's level of profession[19]. For clinicians, the most important aspect of diagnostics for CIN is to differentiate LSIL from HSIL. For this reason, many currently ongoing studies search for new markers that may streamline the diagnostic process[20]. Accurate screening for ≥CIN2 lesions prior to colposcopic triage is key to early screening for cervical cancer. The use of p16/Ki-67 DS to detect cervical precancerous lesions has received attention from investigators for its high sensitivity and specificity[14, 21]. The technique of CB production has been widely used in non-gynecologic pathology, which is mature, simple and easy to operate, and can maximize the enrichment of cells and also obtain morphological details similar to histology such as nests, sheets, papillae and three-dimensional structures of certain solid tissues. CB not only has histological advantages, but also provides sufficient material for subsequent molecular

pathological examinations such as special staining, Immunohistochemical(IHC), PCR and sequencing. The prepared CB can be stored for a long time, while the preparation of CB provides convenience and possibility for the transportation and preservation of samples in the multicenter construction of cervical cancer screening, but its use in cervical cancer screening has been rarely reported in the literature. In this paper, we applied CB made from leftover specimens of LBC combined with p16/Ki-67 DS to compare the role of HR-HPV, DNA ploidy and p16/Ki-67 DS in early screening of cervical cancer, especially before colposcopic triage.

P16, also known as $p16^{INK4a}$, is encoded by the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene located on chromosome 9p21.3. It is a cell cycle protein that regulates cell proliferation in the G1-S phase due to the reciprocal relationship with another tumor suppressor protein-Rb(20). In HPV-infected cells, interference with the pRb-E2F1 pathway by E7 through a negative feedback loop [22, 23]. Induced overexpression and accumulation of p16 in cells. Therefore, p16 is considered as an alternative marker of persistent HR-HPV infection. Cells with heterogeneous proliferation exhibit p16 overexpression, which is easily detected by immunohistochemistry. Since E7 oncogene induces cell transformation of all HR-HPV types, dysplastic cells exhibit p16 overexpression, which is easily detected by immunohistochemistry) [24]. In recent years, P16 immunohistochemical staining has been increasingly recommended to differentiate HSIL from unrelated neoplastic disease, and studies have shown that P16 immunohistochemical staining can be used as a specific biomarker to predict the progression of CIN[25]. Ki-67 is a proliferating cell-associated antigen, also known as MIB-1, that is detected in the nuclei of proliferating cells in all phases of the cell cycle (G1, S, G2, and M), but is largely unexpressed in quiescent cells (G0)[26]. Ki-67 has been shown to play multiple roles in regulating cell cycle progression as a marker of tumor cell proliferation capacity[27]. When CIN occurs, Ki-67 expression gradually extends and spreads from the basal layer to the mid-surface layer, consistent with the extension of CIN. Detection of Ki-67 has been widely used in the ancillary diagnosis of precancerous cervical cancer and cancer[28]. Alshenawy et al. found that Ki-67 expression gradually increased with increasing CIN grade, and in cancerous tissues, Ki67 expression reached 100%. p16 and Ki67 act as tumor suppressors and cell proliferation markers, respectively, and overexpression of p16 and Ki67 are mutually exclusive under normal conditions and do not usually occur in the same cervical epithelial cells[29]. Detection of p16/Ki-67 co-expression can be used as a predictor of HR-HPV cell transformation and high-grade CIN lesions[10]. It has been shown that p16/Ki67 DS can be a more valuable method in detecting cervical lesions when comparing the combination of p16/Ki67 DS test, LBC and HPV test. Wu Y et al. repoerted that the ROC curve for p16/Ki-67 was greater than HC-II in ASCUS, LSIL (0.799 vs 0.696, 0.708 vs 0.531; all 0.05). For patients with cytologic diagnosis of ASCUS, LSIL, the p16/Ki-67 DS method can be an effective method to assist in the diagnosis of high-grade cervical lesions, and the screening efficiency is superior to that of HR-HPV[30]. Excellent performance of p16/Ki-67 DS was observed in one study, especially in women under 30 years of age, with an area under the ROC curve of 0.762 ($p<0.001$)[31]. As described in the previous article the area under the ROC curve for p16/Ki-67 DS on CB in our study was greater than that for HR-HPV and DNA ploidy (0.900 vs. 0.557,0.583), ($p<0.001$).

The morphology of ASC-US cells is characterized by an enlarged nucleus that is 2.5 to 3 times larger than the normal mid-layer nucleus, a mildly increased nucleoplasmic ratio, a mildly deepened nuclear chromatin, and an abnormal nucleus or a strong orangish change in the cytoplasm, which is a risk indication for the presence of lesions, but not a definitive diagnosis of abnormal cells. The diagnostic cytologic presentation of ASC-US is unclear, i.e., it may suggest the occurrence of HPV infection or other malignant lesions in the cervix, or it may represent only benign reactive changes such as inflammation, becoming a gray area for cytologic diagnosis due to its low specificity and poor reproducibility, as well as a currently recognized diagnostic difficulty. Although ASC-US is considered a low-risk cervical cytologic abnormality, about 5-10% of women with primary ASC-US actually have an underlying high-grade cervical lesion (CIN2+ and CIN3+) on histologic diagnosis and need to be followed up[32]. If ASC-US is not effectively triaged, the best time for treatment is missed and the lesion may progress to a higher grade or squamous cell carcinoma. ZHU et al. found that CIN2 + accounted for only 18.0% and CIN 3+ for only 8.3% of ASCUS, and that colposcopy would overtreat ASC-US if all ASC-US patients underwent colposcopy. the total positive rate of HPV-DNA testing in 300 ASC-US cases was 85.3%, and the positive rate of p16/Ki67 immunocytochemistry testing was 32.0%[33]. The overall positive rate of HPV-DNA testing was 85.3% and the positive rate of p16/Ki67 immunocytochemistry was 32.0% in 300 ASC-US cases, indicating that staging ASC-US with p16/Ki67 DS instead of HPV-DNA testing reduced the referral rate for colposcopy by 53.3%.

According to the National Comprehensive Cancer Network (NCCN) guidelines for cervical cancer screening, the treatment of LSIL cases is based on clinical observation. The progression rate of p16/Ki67 positive cases and negative progression rate of 30.8% and 4.3%, respectively, in the study of Vrdoljak-Mozetic et al. It were significantly higher than the progression rate of negative cases, confirming the predictive role of the p16/Ki67 DS assay for LSIL case regression[34]. A European study including 27,349 women[13]. Similarly, p16/Ki67 DS showed a high positive predictive value for detecting HSIL cases from LSIL cases. Most of the LSIL can regress or heal on their own, and only a small proportion is at risk of progressing to higher grade lesions. Since clinical obstetricians and gynecologists in different hospitals interpret these patients differently their clinical management and follow-up are somewhat divergent therefore, how to manage patients with ASC-US/LSIL cervical cytology more effectively is becoming more and more important. Clinical intervention and treatment of cases with a cytologic diagnosis of ASC-US/LSIL remain in limbo. It is important to implement effective triage methods for ASC-US and LSIL cytology findings to identify women at highest risk for potential precancerous lesions and those requiring immediate further diagnostic follow-up[35]. In our study, it was demonstrated that the area under the AUC curve, NRI index, and IDI index were significantly increased for p16/Ki-67 DS on CB compared to HR-HPV and DNA ploidy, verifying that p16/Ki-67 DS on CB significantly improved the correct classification of patients with ≥CIN2 and was a good method for triaging ASC-US/LSIL patients.

The diagnostic challenge in cervical cancer screening is cervical glandular lesions, and it is difficult to distinguish between hyperplasia and neoplasia. One study showed that p16/Ki-67 DS was positive in 92.5% of 40 cases of cervical adenocarcinoma and only 1 of 16 cases of cervical tissue without glandular lesion was positive for DS, suggesting that p16/Ki-67 DS is a potential tool to diagnose cervical

glandular lesions[36]. In this study, DS was also positive for adenocarcinoma and squamous cell carcinoma, but due to the small number of cases, further analysis will be made after the expansion of cases in the future.

The clinical application of p16/Ki-67 DS in combination with cervical liquid-based CB technology maximizes the benefits of cervical cancer screening while reducing and avoiding unnecessary waste of resources and potential harm caused by misdiagnosis and overdiagnosis, and helps to achieve cervical lesion interpretation, prediction, and improve the diagnostic efficacy of ≥CIN2. In practice, it has very good efficacy for stratified management of ASC-US/LSIL patients, detection rate and avoidance of unnecessary biopsies and overtreatment, providing an effective test in cervical cancer screening triage management. This will benefit women by reducing the psychosocial impact of having repeated abnormal smears and undergoing colposcopy, while also reducing the cost of testing associated with colposcopy. It also has the advantages of being easy to repeat, objective and efficient. There is good agreement with histopathology, which can assist in pathologic diagnosis and determination of clinical outcome. The p16/Ki-67 DS on CB is suggested to be a good tool to triage ASCUS, but more research is needed to support this view.

Limitations of the study

In this experiment, there are also limitations, which are summarized as follows: 1. The sampling should be done as standardized as possible to ensure a certain pressure on the site taken to obtain a sufficient amount of cells. 2. 8 of the 58 patients in our study with histologically confirmed ≥CIN2 were negative for p16/Ki-67 DS, and repeat review revealed histologically small focal CIN2 involving glands. 3. For deep focal lesions in the cervical canal, the superficial location of the sampling leads to missing deep lesions, and Endocervical curettage (ECC) can resolve such false negatives. 4. The clinician should avoid menstruation and try to remove mucus and blood before sampling to obtain high quality CB. 5. Routine sampling does not take the vaginal wall, resulting in easy to miss lesions in the vaginal wall, and it is debatable whether to do additional sampling of the vaginal area. The CB were leftover LBC specimens with relatively reduced cell volume, which may also cause false-negative results.

Abbreviations

DS:double staining ; LBC:liquid-based cytology; IHC：Immunohistochemical; CIN: Cervical intraepithelial neoplasia; NRI:Net reclassification improvement; IDI:Integrated discrimination improvement index; LSIL:Low-grade squamous intraepithelial lesion；HSIL:High -grade squmnous intraepithelial lesion.

Declarations

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Author contributions

XS and YG designed the study. YG conducted study selection and quality control. FM collected data. XS validated data. YG and FM performed statistical analysis. YG reviewed the reliability of the results. XS prepared the manuscript and figures. XS and YG were involved in manuscript revision and editing. All authors reviewed and approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the submission. The raw data are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of Haidian Maternal and Child Health Hospital, Beijing, China. The approval number is 2021-2.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

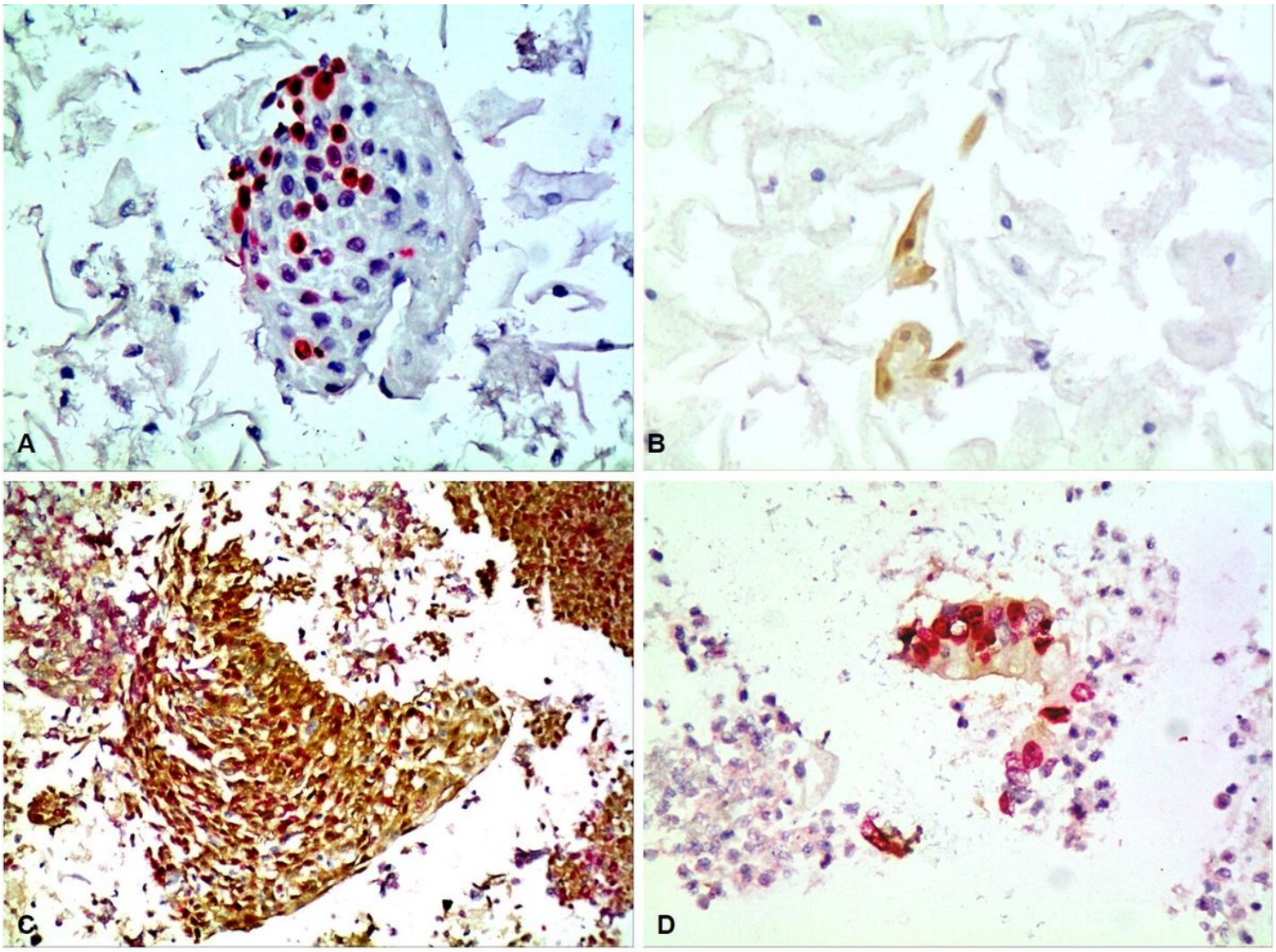

Figure 1

p16/Ki-67 immunohistochemical double staining on CB.(A)Immunohistochemical double staining on CB showed Ki-67 staining alone, red nuclei only, p16Ki-67 double staining negative.Magnification×400.(B)CB immunohistochemistry double stained p16 alone, cytoplasm and nucleus were brown, p16Ki-67 double stained negative.Magnification ×400.(C)The biopsy result was squamous cell carcinoma, CB showed lamellar squamous epithelium with positive p16Ki-67 double staining.Magnification×200.(D)Biopsy showed adenocarcinoma of the uterine cervix with CB showing adenoid structures, mucus-rich cytoplasm and positive p16Ki-67 double staining.Magnification×400.

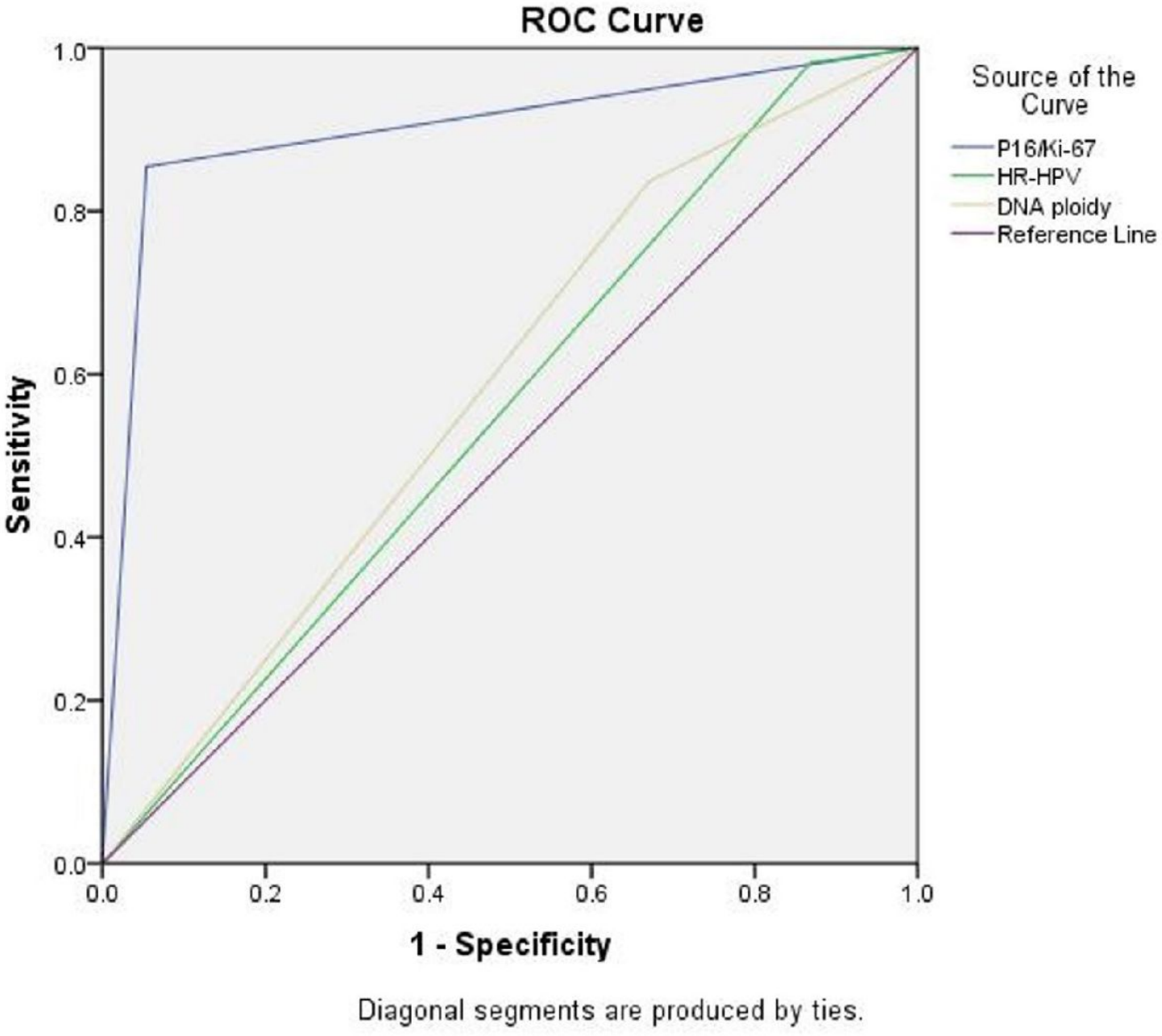

Figure 2

ROC curves plotted for the three screening methods

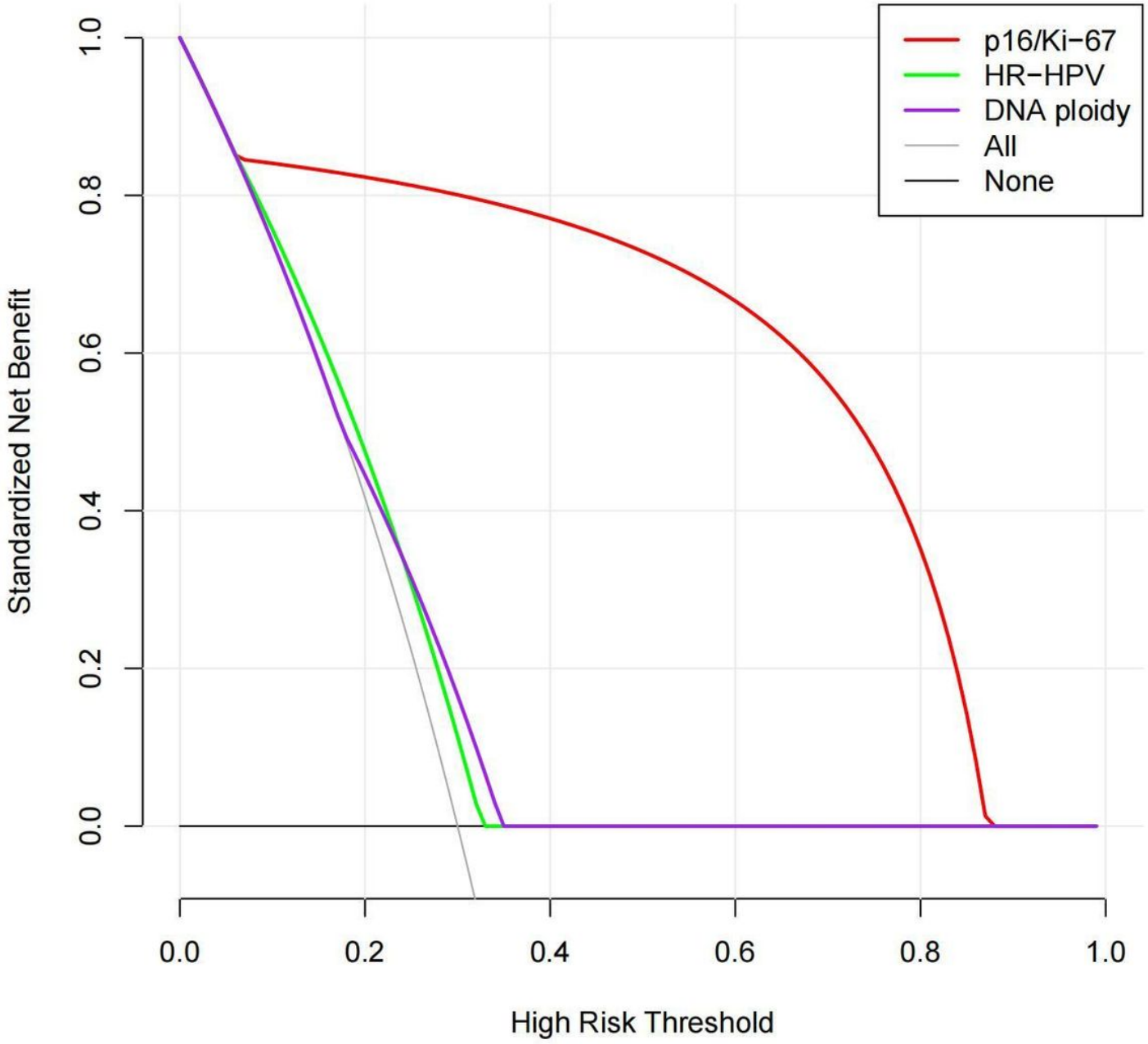

Figure 3

Decision curve (DCA) for different diagnostic indicators