

The value of p16^{INK4a} immunostaining for high-grade squamous intraepithelial lesions in human papillomavirus–negative patients

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Abstract

Background : This study aims to evaluate the value of p16 INK4a immunostaining for high-grade squamous intraepithelial lesions in human papillomavirus–negative patients in Beijing, China.

Methods: In this study, we evaluated the value of p16 INK4a immunostaining, as well as cytology and colposcopy, for predicting high-grade squamous intraepithelial lesions (HSIL) in human papillomavirus (HPV)-negative patients by comparing the methods with the haematoxylin and eosin (H&E) staining pathological diagnosis of HPV-negative patients.

Results: Of 122 patients negative for the high-risk HPV (HR-HPV) subtype, 26 (21.3%) underwent colposcopically directed multiple punch cervical biopsy with a H&E pathological diagnosis of HSIL and above (HSIL+), 11 patients (9.0%) had cervical intraepithelial neoplasia (CIN)2, nine patients (7.4%) had CIN3, and six patients (4.9%) had infiltrating carcinoma. Cytology, colposcopy, and p16 INK4a immunostaining had 52.4%, 38.5%, and 92.3% sensitivity, respectively, and 76.2%, 94.8%, and 99% specificity, respectively. The positive predictive value of cytology, colposcopy, and p16 INK4a immunostaining was 31.4%, 66.7%, and 96%, respectively, and the negative predictive value was 88.5%, 85.1%, and 97.9%, respectively. Compared with H&E staining, the kappa of cytology, colposcopy, and p16 INK4a immunostaining was 0.327, 0.323, and 0.926, respectively.

Conclusion: Positive p16 INK4a immunostaining is very strongly consistent with an H&E diagnosis of CIN2+, and it can be used as an objective detection index for HSIL+ diagnosis of HPV-negative patients with CIN2+.

Background

Cervical cancer is the third most common malignancy in women worldwide.¹ Much epidemiological data proves that invasive carcinoma of the cervix is closely related to persistent infection by the high-risk human papillomavirus (HR-HPV) subtype.² Regular cervical cancer screening for women of reproductive age can reduce the morbidity and mortality rates of cervical cancer. Cytological screening is used for cervical cancer screening but faces disadvantages such as low sensitivity and a lack of cytologists. Based on cervical cancer screening of the population in recent years, many studies have found that both combined screening with cytology + HR-HPV subtype detection and preliminary screening with HR-HPV subtype alone enhances the detection rate of the screening.³

The management of women with negative HPV detection and the identification of an objective index that can reveal the presence of a lesion and the lesion grade are critical. Recent studies have mainly focused on finding alternative biomarkers of cervical cancer. Furthermore, diagnosis for suspected cervical lesion with negative HR-HPV subtype detection and women with invasive carcinoma of the cervix currently mainly depends on pathological diagnosis, but morphological diagnosis with haematoxylin and eosin (H&E) staining alone is greatly affected by individual difference and the evaluations of different

pathologists on lesions of the same grade are inconsistent, particularly when diagnosing cervical intraepithelial neoplasia (CIN).

The tumour suppressor protein p16^{INK4a} is a cyclin-dependent kinase (CDK) inhibitor that is inactivated in many cancers. This inactivation leads to the inactivation of the retinoblastoma protein (Rb). However, in HPV-associated tumours, the HPV E7 protein will combine with Rb, and inactivate it. In this process, p16^{INK4a} levels increase markedly.⁴ Currently, positive p16^{INK4a} immunostaining can be used as a marker of high-grade intraepithelial lesions (HSIL).⁵ At present, p16^{INK4a} immunostaining is widely used for the precise diagnosis of HPV-related diseases, especially those patients with CIN2.^{5,6} However, there is insufficient research on p16^{INK4a} in HPV-negative patients. Therefore, we conducted this study to evaluate the value of p16^{INK4a} immunostaining for high-grade squamous intraepithelial lesions in human papillomavirus-negative patients

Methods

1.1 Subjects

This was a retrospective study. From January 2014 to December 2014, patients who were negative for the HR-HPV subtype were recruited in this study to evaluate the value of p16^{INK4a} immunostaining for detecting HSIL and above (HSIL+). These patients had undergone colposcopy and colposcopically-directed multiple punch cervical biopsy at our hospital. In our study, even cases with HPV neg/cytology neg underwent colposcopy because these patients had several clinical symptoms. All biopsy tissues underwent pathological examination. This study was conducted in accordance with the Declaration of Helsinki and approved by the Human Ethics Committee of Peking University First Hospital. All methods were carried out per cervical cancer screening guidelines. All eligible participants provided written, informed consent to be included in this study.

1.2 Inclusion and exclusion criteria

Inclusion criteria: (1) patients who were negative for the HR-HPV subtype; (2) older than 18 years of age; (3) patients who have signed informed consent. Exclusion criteria: (1) patients who were pregnant or nursing women; (2) patients whose data were incomplete.

1.3 Cytological detection

A liquid-based, thin-layer cytologic preparation was used, and the 2001 Bethesda System (TBS) was used for diagnosis. Tissues were evaluated as within normal limits (WNL), atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells – cannot exclude high-grade squamous intraepithelial lesion (ASC-H), a low-grade squamous intraepithelial lesion (LSIL), HSIL, squamous cell carcinoma (SCC), and atypical glandular cells (AGC).

1.4 HPV detection

HPV was tested using the *digene* Hybrid Capture 2 (HC2) High-Risk HPV DNA Test (QIAGEN, Gaithersburg, MD, USA) with the Rapid Capture System (QIAGEN), which is based on signal amplification using RNA probes to target the entire HR-HPV genome.⁸ All steps were performed according to the manufacturer's protocols. Briefly, cervical brush samples collected in preserve cytological solution underwent denaturation, hybridisation, capture, and amplification of chemiluminescent signal detection. We also used the HybriMax HPV blot, which captures 21 HPV genotypes: namely six low-risk types (HPV 6, 11, 42, 43, 44, and CP8304) and 15 HR types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) that are common in the Chinese population.⁹ The HC2 test was used for 43 patients, and the HPV blot was used for 74 patients.

1.5 Colposcopy

Patients with cytological LSIL+ and all patients with AGC underwent colposcopy. Patients with no cytological abnormality or with HPV-negative ASC-US, but with suspected clinical symptoms (such as contact bleeding, irregular vaginal bleeding, increased vaginal discharge, etc.) of cervical cancer, also underwent colposcopy. Colposcopy was carried out per standard procedures. We carried out colposcopically-directed multiple punch cervical biopsy for the most abnormal part of the suspected lesion. Cervical four-quadrant randomised biopsy and endocervical curettage were used when the colposcopy was unsatisfactory.

1.6 Pathological diagnosis of cervical biopsy samples

We used a three-level classification method for the pathological H&E-stained sections of CIN1, CIN2, and CIN3. CIN1 was considered LSIL; CIN2 and CIN3 were classified as HSIL.

1.7 Detection of p16^{INK4a} protein and evaluation of positive immunostaining results The immunohistochemical method was adopted for detecting p16^{INK4a}. Paraffin sections of cervical tissue were stained according to the reagent kit instructions (Ventana Medical System Inc, Arizona USA). We used 1:100 dilution of the primary antibody (mouse anti-human p16^{INK4a} monoclonal antibody; clone number E6H4, USA). The primary antibody was replaced with phosphate buffer solution (PBS) to construct the negative control; known p16^{INK4a}-positive pancreas sections were used as the positive control.

Cells with positive p16^{INK4a} immunostaining had brownish-yellow nuclei and cytoplasm. We determined the staining grade according to the percentage of p16^{INK4a}-positive cells: positive, epithelial diffuse layer staining; focal positive, focal, discontinuous positive staining; negative, no obvious staining.

1.8 Cervical loop electrosurgical excision procedure (LEEP)

For patients with H&E pathological diagnosis of CIN2 and above (CIN2+), cervical LEEP was carried out in the next menstrual cycle, and the obtained sample was once again pathologically diagnosed.

1.9 Statistical analysis

We used the software program SPSS 13.0 (SPSS, Chicago, IL, USA) to conduct the statistical analysis. The continuous variables of normal distribution were expressed as mean \pm standard deviation, the continuous variables of non-normal distribution were expressed as a median (interquartile range[IQR]), and the categorical variables were expressed as a frequency (percentage[%]). For two comparisons, each value was compared by t-test. For multiple comparisons, each value was compared by one way ANOVA following Dunnett test when each datum conformed to a normal distribution, while the non-normally distributed continuous data were compared using non-parametric tests. The chi-square test tested the counting data. A value of $P < 0.05$ was considered statistically significant.

Results

2.1 The general characteristics

A total of 122 HR-HPV-negative patients were included in this study. The age of these participants ranged from 19 years to 77 years old. The average age was 42.45 ± 11.33 years. Table 1 lists the details.

2.2 Cervical biopsy pathological results

Of the 122 patients, 76 patients (62.3%) had the pathological result of inflammation, 20 patients (16.4%) had CIN1, 11 patients (9.0%) had CIN2, nine patients (7.4%) had CIN3, and six patients (4.9%) had SCC. In total, 26 patients (21.3%) were diagnosed with CIN2+.

2.3 Value of cytology for detecting HSIL

We divided the cytology screening results into low-grade abnormality (WNL, ASC-US, LSIL) and high-grade abnormality (ASC-H, HSIL, AGC). Of the 26 patients with CIN2+, 12 patients (46.2%) were in the low-grade abnormality group, and 14 patients (53.8%) were in the high-grade abnormality group. CIN2+ detection between the cytology and cervical H&E pathological results was statistically significantly different (Table 2). The κ value for high-grade abnormality for the cytology and pathological diagnosis of CIN2+ was 0.327 ($\chi^2 = 13.173$, $P = 0.001$).

2.4 Value of colposcopy for detecting HSIL

Of the 122 HR-HPV-negative patients who underwent colposcopy, 62 patients (50.8%) were diagnosed as WNL, 45 (36.9%) were diagnosed as LSIL, 11 (9.0%) were diagnosed as HSIL, and four (3.3%) were diagnosed with infiltrating carcinoma. We divided the colposcopy results into low-grade abnormality (WNL and LSIL) and high-grade abnormality (HSIL and infiltrating carcinoma). Eighteen patients (14.8%) had a high-grade abnormality. There was a statistical difference for CIN2+ detection by colposcopy and H&E pathological results (Table 3). The κ value of the high-grade abnormality for the colposcopy and pathological diagnosis of CIN2+ was 0.323 ($\chi^2 = 13.164$, $P = 0.001$).

2.5 Value of p16^{INK4a} immunostaining for detecting HSIL

Of the 122 HR-HPV-negative patients that underwent p16^{INK4a} immunostaining, 25 (20.5%) had positive staining, 20 (16.4%) had focal positive staining, and 77 (63.1%) had negative staining. We divided the p16^{INK4a} immunostaining results into low-grade abnormality (negative and focal positive staining) and high-grade abnormality (positive staining). There was a statistical difference for CIN2+ detection by p16^{INK4a} immunostaining and H&E pathological results (Table 4). The kappa of the positive p16^{INK4a} immunostaining and pathological diagnosis of CIN2+ was 0.926 ($\chi^2 = 104.59$, $P = 0.000$).

2.6 Lower Anogenital Squamous Terminology (LAST) project HSIL diagnosis of cervical biopsy tissue

In LAST, the diagnostic terminology for different parts of the anus and reproductive tract, and the pathological diagnosis of cervical tissue are consistent with TBS terminology¹⁰⁻¹¹. In this study, classification based on cytology, colposcopy, and H&E pathology detected 26 cases of HSIL+ (CIN2+) (Figure 1); based on LAST, there were 24 cases of HSIL+. The differences were the two cases that were focally positive for p16^{INK4a}. After cervical LEEP, one case was diagnosed as CIN1, i.e. LSIL; another was diagnosed as CIN2, i.e. HSIL. The 2 high grade lesions were not considered in the LAST.

2.7 Pathological results of CIN2+ after cervical LEEP

All 26 patients with H&E pathological diagnosis of CIN2+ underwent cervical LEEP, of which 24 had positive p16^{INK4a} immunostaining, and two patients had focal positive p16^{INK4a} immunostaining. The latter two patients underwent cervical LEEP, and returned the pathological result of CIN1 and CIN2, respectively. Of the total 122 patients included in the study, 25 were p16^{INK4a}-positive: one patient with punch biopsy findings of CIN1 is currently under follow-up; the remaining 24 underwent cervical LEEP, and the LEEP specimen pathological results were CIN1 (three patients), CIN2 (nine patients), CIN3 (six patients), and infiltrating carcinoma (six patients). There was a statistically significant difference between the punch biopsy pathological results under colposcopy and the pathological results after cervical LEEP ($\chi^2 = 31.704$, $P = 0.000$) (Table 5). The positive predictive value (PPV) of positive p16^{INK4a} expression for pathological HSIL+ after cervical LEEP was 87.5%.

2.8 Comparison of detection methods for cervical precancerous lesions

In summary, H&E staining was the gold standard for detecting cervical precancerous lesions. We evaluated cytology, colposcopy, and p16^{INK4a} immunostaining for detecting cervical HSIL in cervical HPV-negative patients (Table 6). The sensitivity, specificity, PPV, and negative predictive value (NPV) of p16^{INK4a} immunostaining were all >90%, which was significantly better than that of cytology and colposcopy. Including focally positive p16^{INK4a} immunostaining in the detection of cervical precancerous lesions increased the sensitivity and NPV to 100%, but greatly reduced the specificity and PPV, meaning it is not the best choice.

Discussion

Numerous epidemiologic studies have proven that persistent HR-HPV infection is the main causative factor of HSIL and infiltrating carcinoma. HR-HPV infection can be detected in almost all patients with precancerous lesion of the cervix and invasive carcinoma of the cervix, and there would be positive clinical detection of an HR-HPV subtype. However, a certain proportion of patients with precancerous lesion of the cervix and invasive carcinoma of the cervix may have negative HR-HPV subtype detection, and the management of such patients should be investigated further in particular. At present, HR-HPV detection enhances screening sensitivity when it is used as an objective index for cervical cancer screening (Early screening or combined cytological screening).^{2, 12-13} However, there is currently a lack of more sensitive and objective screening diagnosis indexes for HR-HPV-negative women.

Previous study have found that the reproducibility of H&E staining morphological diagnoses by pathologists is poor and that the consistency of CIN2 diagnosis is <50%.¹⁴ The reproducibility of CIN2+ morphological diagnosis in the ASCUS/LSIL Triage Study for Cervical Cancer (ALTS) was only 43%.¹⁵ In another study, two pathologists agreed with 84% and 81%, respectively, for CIN3 diagnosis; for CIN2, the agreement was 13% and 31%, respectively.¹⁶ There are vast differences among pathologists for H&E staining diagnosis of CIN2, and there are many false positive or false negative results. Recent studies have suggested that auxiliary use of immunohistochemical staining may aid the accuracy of CIN2 diagnosis.

The p16^{INK4a} protein can compete with cyclin D1 to bind with CDK4, inhibiting CDK4 activity. The p16^{INK4a}/CDK-cyclin D/Rb complex is the key factor when cells exit the G1 phase of the cell cycle and enter the S phase. Any gene abnormality in this molecular chain may result in the loss of control of molecular action on the cell cycle. The E7 protein expressed by HR-HPV interferes with the normal function of the *RB* gene. E7 binds with phosphorylated Rb (pRb), inactivating the function of the *RB* gene, eliminating the negative feedback inhibition of pRb on p16^{INK4a} protein expression, and results in p16^{INK4a} overexpression.⁴ This leads to disorder of the cell cycle of cervical epithelial cells, resulting in the characteristic of immortality and the initiation of a series of carcinogenesis processes.¹⁷⁻²⁰

Li et al. from our department carried out p16^{INK4a} and Ki-67 immunostaining on the pathological sections of patients with CIN who were aged <35 years. These researchers found that p16^{INK4a} and Ki-67 immunostaining had very good consistency with CIN grading.²¹ Galgano et al. found that sensitivity was 86.7% and specificity was 82.8% for CIN2+ and that p16^{INK4a} immunostaining is a useful and reliable diagnostic adjunct for distinguishing biopsies with and without CIN2+.²² Bergeron et al. found that p16^{INK4a}-immunostained slides significantly increased the diagnostic accuracy for detecting high-grade CIN as compared with H&E slides and that the reproducibility of p16^{INK4a} immunostaining interpretation was excellent²³. Other investigators suggested that the conjunctive use of H&E morphology with p16^{INK4a} immunostaining improved inter-observer agreement of the CIN2+ diagnosis.²⁴ The LAST Project working group of the College of American Pathologists and ASCCP proposed that the addition of

p16^{INK4a} immunostaining in some cases may provide a more reliable and consistent pathological interpretation.^{10, 25} However, can p16^{INK4a} be used as a molecular biological substitute for pathological HSIL of the cervical tissues in HR-HPV-negative patients? Zhang et al. found that diffuse p16 immunostaining is the hallmark of HSIL because it correlates 100% with CIN2 and CIN3 lesions between initial biopsy and cervical LEEP specimens, whatever the HPV status.²⁶ Solano et al. found that p16^{INK4a} immunostaining had more diagnostic benefits, where their retrospective study of 596 patients revealed HSIL/CIN2-3 not found in the initial H&E staining.²⁷

There was very strong consistency between positive p16^{INK4a} immunostaining and H&E staining pathological diagnosis in our study (kappa = 0.926). The PPV and NPV of high-grade lesion diagnosis was 92.3% and 97.9%, respectively. Almost 100% of the cervical HSIL or infiltrating carcinoma could be excluded for patients with negative p16^{INK4a} immunostaining. For the follow-up of the pathological results, we found that the PPV of p16^{INK4a}-positive staining for pathological HSIL+ after cervical LEEP was 87.5%, which is higher than that reported by Clinton et al., who found that HSIL detection increased from 48% to 76% ($P < 0.05$) after the wide clinical application of p16^{INK4a} immunostaining.¹¹ Besides, in our study, two patients with H&E-diagnosed CIN2 had focal positive p16^{INK4a} immunostaining. The diagnoses of these two patients would be classified as LSIL according to the recommendations of the 2012 LAST guidelines, and follow-up may be conducted for management. The two patients also underwent cervical LEEP in the next menstrual cycle after cervical biopsy, and the results were CIN1 and CIN2, respectively.

Limitations. This study had several limitations. Firstly, this trial was not a randomised controlled trial. Secondly, this study was only a single-centre trial and these 122 HPV negative cases was limited which could not represent the whole patents in our country. Another multiple center trial with large sample size was still needed in the future. Thirdly, diagnostic cervical LEEP can be conducted for elderly women, women with persistent CIN2 for >2 years, or patients with other risk factors. As there were only two such patients in this study, more cases should be gathered in the future for an in-depth study to facilitate suggestions for suitable management. Fourthly, it will be better if patients obtained a double negative using both the methods employed to confirm negativity, but as a retrospective study, our patients only received the HPV DNA assays in our hospital which should be further research. Besides, although included samples were HPV negative that the methods can not rule out infection with a genotype not included within the assays used.

Conclusion: Immunostaining for p16^{INK4a} can be used as a molecular biological substitute for evaluating pathological HSIL of cervical tissue in HR-HPV-negative patients and can be used to aid HSIL detection.

List Of Abbreviations

HSIL: high-grade squamous intraepithelial lesions

HPV: human papillomavirus

H&E: haematoxylin and eosin

HR-HPV: high-risk HPV

CIN: cervical intraepithelial neoplasia

CDB: Colposcopically directed cervical biopsy

H&E:haematoxylin and eosin

CDK:cyclin-dependent kinase

HSIL:high-grade intraepithelial lesions

TBS:Bethesda System

WNL:within normal limits

ASC-US: atypical squamous cells of undetermined significance

ASC-H:atypical squamous cells – cannot exclude high

LSIL: low-grade squamous intraepithelial lesion

SCC: squamous cell carcinoma

AGC: atypical glandular cells

HC2: Hybrid Capture 2

PBS:phosphate buffer solution)

LEEP: loop electrosurgical excision procedure

IQR: interquartile range

ASCCP: American Society for Colposcopy and Cervical Pathology

PPV: positive predictive value

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Peking University First Hospital.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Competing interests

All of the authors had no any personal, financial, commercial, or academic conflicts of interest separately.

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Not applicable.

Authors' contributions

ZD and SJ conceived of the study, and ZXS participated in its design and coordination and HB helped to draft the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1
Distribution of patient age.

Age (years)	≤25	25-34	35-44	45-54	55-64	≥ 65
Percentage	3.3%	22.1%	35.2%	23.8%	12.3%	3.3%
	(4/122)	(27/122)	(43/122)	(29/122)	(15/122)	(4/122)

Table 2
Comparison of cytology results and cervical biopsy pathological results.

Pathological result	Inflammation		CIN1	CIN2	CIN3	SCC	Total	χ^2	P
	n	%							
Cytology results									
WNL	37	78.7	6	12.1	12.1	24.3	47		
ASC-US	14	66.7	2	29.5	29.5	14.8	29.5	21	
LSIL	10	52.6	3	63.1	62.1	10.5	15.3	00.0	19 38.2750.008
ASC-H	6	46.2	4	30.8	30.0	3	23.1	100.0	13
HSIL	5	29.4	2	11.8	52.9	4.3	17.6	211.8	17
AGC	4	80.0	0	0.0	0.0	0.0	0.0	0.0	5

Table 3
Comparison of colposcopy results and cervical biopsy pathological results.

Pathological results	Inflammation		CIN1	CIN2	CIN3	SCC	Total	χ^2	P
	n	%							
Colposcopy results									
WNL	44	75.9	6	10.3	58.6	7	12.1	100.0	62
LSIL	30	66.7	11	24.4	24.4	12.2	12.2	45	70.4
HSIL	2	18.1	2	18.1	43.6	40.0	0	327.3	11
Carcinoma	0	0.0	1	25.0	0.0	125.0	25.0	0.4	

Table 4
Comparison of p16^{INK4a} immunostaining results and cervical biopsy pathological results.

Pathological results	Inflammation		CIN1	CIN2	CIN3	SCC	Total	χ^2	P
	n	%							
p16 ^{INK4a} immunostaining results									
Negative	69	89.6	8	10.4	0.0	0.0	0.0	77	
Focal positive	7	35.0	11	55.0	21.0	0.0	0.0	20	134.76
Positive	0	0.0	1	4.0	916.0	936.0	624.0	25	

Table 5

Comparison of the pathological results before and after cervical LEEP in p16^{INK4a}-positive patients.

Colposcopically directed cervical biopsy pathological results	Pathological results after cervical LEEP						Total		
	CIN1		CIN2		CIN3			Infiltrating carcinoma	
	n	%	n	%	n	%			
CIN2	3	33.3	5	55.6	1	11.1	0.0	9	
CIN3	0	0.0	4	44.4	5	55.6	0.0	9	
Infiltrating carcinoma	0	0.0	0	0.0	0	0.0	6	100.0	6
Total	3	12.5	9	37.5	6	25.0	6	25.0	24

Table 6

Comparison of different methods for detecting cervical precancerous lesions.

Parameter	Cytology	Colposcopy	p16 ^{INK4a} immunostaining (positive staining)	p16 ^{INK4a} immunostaining (positive and focal positive staining)
Sensitivity	52.4	38.5	92.3	100
Specificity	76.2	94.8	99.0	80.2
PPV	31.4	66.7	96.0	57.8
NPV	88.5	85.1	97.9	100
Kappa with H&E staining	0.327	0.323	0.926	

Figures

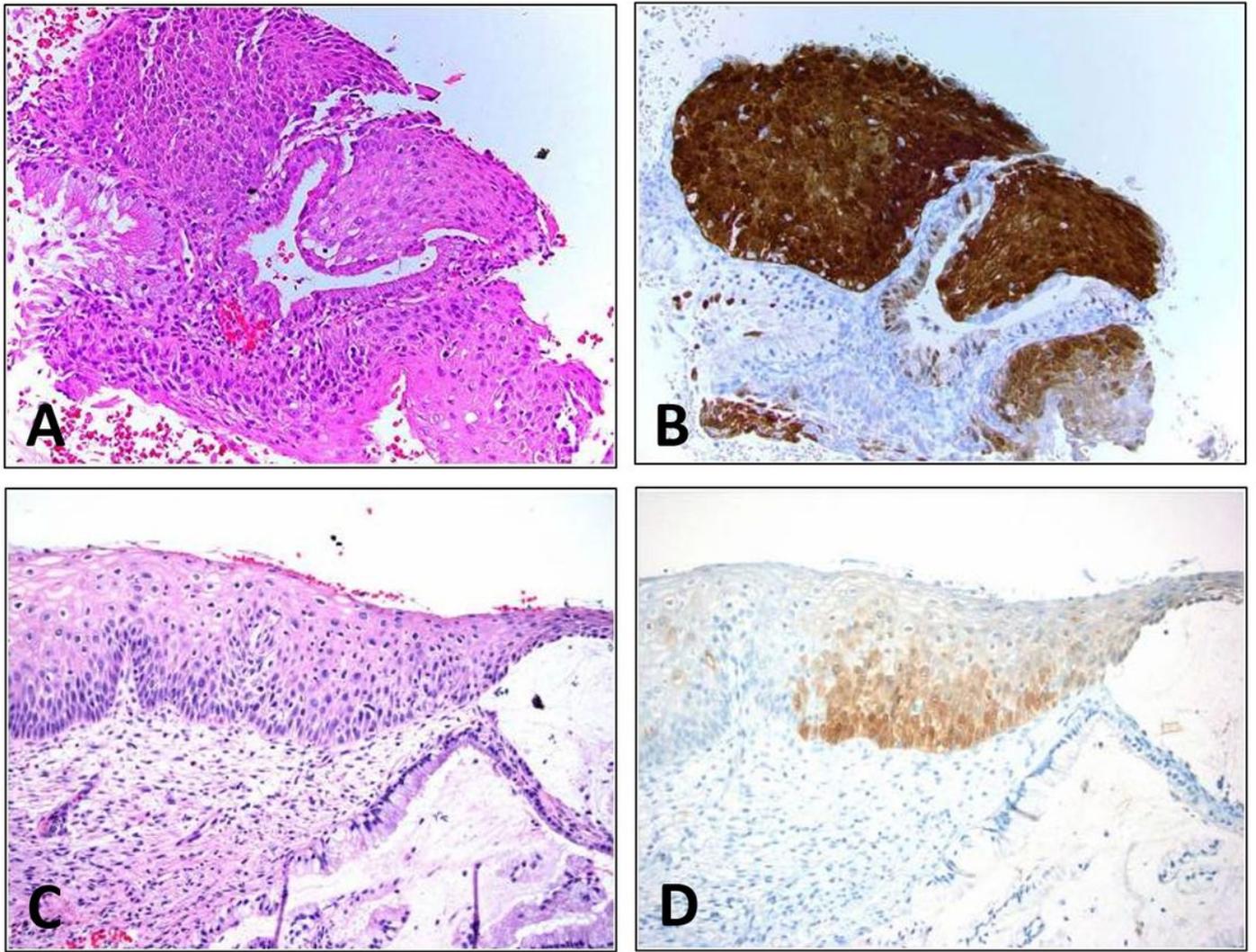


Figure 1

The H&E staining, cytology, colposcopy, and p16INK4a immunostaining for detecting cervical HSIL in cervical HPV-negative patients. A: The H&E staining; B: cytology; C: colposcopy; D: p16INK4a immunostaining.