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Differential diagnosis of high-grade squamous intraepithelial lesions and benign atrophy in older women using p16 immunocytochemistry

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ABSTRACT

Background: For cervical cancer screening, routine cytology has a high specificity but a lower sensitivity. In older women, atrophy, which may mimic HSIL, presents a diagnostic challenge. p16 is a widely used biomarker for histological diagnosis of HSIL. Our objective was to evaluate PathCIN® $p16^{INK4a}$ immunocytology in identification of high grade dysplasia *vs.* benign atrophy.

Methods: As part of a multi-center screening program, 3351 women were co-tested by p16 immunocytology. Among women referred for colposcopy on basis of cytology and high-risk HPV status, those with atrophy were older than the population screened (52 *vs.* 43 years). Cases from older women with atrophy (n = 116) and controls without atrophy (n = 47) were identified by re-examination of Pap smears. The detection of CIN2+ was compared for p16, cytology and HR-HPV results.

Results: The sensitivity of routine cytology (≥LSIL) was much lower for cases with atrophy (17%) than non-atrophic cases (75%). The sensitivity of p16 immunocytology and of HR-HPV testing was high (88%–100%) both with and without atrophy. The specificity of routine cytology (≥LSIL) was higher for cases with atrophy (79%) than non-atrophic cases (38%). The specificity of p16 immunocytology was high (88–95%) and the specificity of HR-HPV testing was low (31%–33%) both with and without atrophy. Combining p16 with HPV testing and/or routine cytology had no benefit, as compared to p16 staining alone.

Conclusions: p16 immunocytology compares favorably with routine cytology and HPV testing in the differential diagnosis of HSIL and benign atrophy. It is more sensitive than cytology for atrophic specimens, and is more specific than HPV testing. p16 immunocytology may decrease the need for colposcopy referrals and could be a useful tool for early detection of cervical cancer in peri- and post-menopausal women, who are more likely to have HSIL coexisting with atrophy.

1. Introduction

The incidence of cervical cancer in China is increasing rapidly, in contrast to decreasing trends in many Western countries.[1–3] Population-wide cervical cancer screening, which is effective in reducing cervical cancer incidence and mortality, has only been available since 2009, with a lower population coverage than other countries.[3,4] Liquid-based cytology (LBC) and testing for high-risk HPV (HR-HPV) are the most common cervical screening tools. Routine cytology has a high specificity but a lower sensitivity. Testing for high-risk HPV, on the other hand, has a high sensitivity but a low specificity, which can lead to unnecessary colposcopy referral and treatment, increases health costs and causes anxiety for women involved.[5] Cervical cancer screening could be improved by developing biomarkers of higher specificity and sensitivity for detection of high-grade squamous intraepithelial lesions (HSIL).

Atrophic cytology, which is common in Pap smears from

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postmenopausal and perimenopausal women, presents a diagnostic challenge.[6–9] Atrophy can mimic HSIL due to cells lacking maturity and with a high nuclear to cytoplasmic ratio and can lead to an over-interpretation of dysplasia. However, sometimes a high grade or malignant smear might be under-diagnosed as atrophy.[6] Since many women in China are older at their first Pap test than in other countries, atrophy, which may coexist with dysplasia or neoplasia, makes diagnosis particularly challenging. Short-term estrogen treatment, followed by a repeat of the Pap smear can correct false-positive interpretations,[10] but it would be preferable to have biomarkers that can increase the specificity of the initial Pap smear.[11]

One useful biomarker for diagnosis of HSIL is $p16^{INK4a}$ (henceforth p16).[12–14] p16 immunohistochemistry is widely used to facilitate the diagnosis of HPV-associated lesions.[12] p16 is a cyclin dependent kinase inhibitor that regulates the cell cycle. It is expressed at very low concentrations in normal cells, but is strongly overexpressed when *RB* is inactivated by the HPV E7 oncoprotein15. There have been a number of studies evaluating the use of p16 immunocytology in Pap smears,[3,16–22] but few that focus on cases with atrophy.[23] Therefore, our objective was to evaluate the use of p16 immuno-cytology in identification of high-grade dysplasia *vs.* benign atrophy.

2. Materials and methods

2.1. Study population

Between May 2016 and March 2018, 1101 women, aged 42.1 ± 7.4 years, underwent cervical cancer screening via LBC/HPV co-testing in Shenzhen, China. An additional 2250 women aged 44.1 ± 7.1 years, underwent cervical cancer screening via LBC/HPV co-testing in Wuhan, China as a part of the Chinese Multi-Center Screening Trial (CHIMUST). At the time of screening, an additional cytological specimen was placed in the p16 cell preservation solution (Senying Biotechnology Co., LTD., Shenzhen, China) for p16 detection. This study was approved by the ethics committee of Peking University Shenzhen Hospital (BDSY2017 [HYPHEN]005). Informed consent and publication agreement were obtained from all the patients recruited.

Among the 3351 women screened, 10.7% were positive for HR-HPV, 4.4% had a cytological diagnosis of ASCUS, and 5.2% had a cytological diagnosis of >LSIL. CIN2/CIN3 was identified in 58 cases, or 1.75% of the screening population. In both study sites (Shenzhen and Wuhan), women were offered colposcopy if they were (i) positive for HPV16/18, or (ii) positive for other HR-HPV types with a cytology diagnosis ≥ ASCUS, or (iii) with a cytology diagnosis greater than ASCUS regardless of HR-HPV status. Directed and random biopsies plus endocervical curettage were performed under colposcopy.

The study population was created from the colposcopy population. There were 435 cases with histopathology and results for LBC, HR-HPV detection, and histopathology, including 284 women of ≥45 years old. The presence or absence of atrophy was determined by re-examination of Pap smears by two experienced cytopathologists. Atrophic specimens from older women (n = 116, age 51.9 ± 4.3 years) were evaluated by p16 immunocytology without knowledge of histopathological diagnoses. As controls, 47/168 non-atrophic specimens (age 49.8 ± 3.1 years) were chosen without regard to histological diagnosis and examined by p16 immunocytology.

2.2. Liquid based cytology, HR-HPV, p16 immunocytology, and histological diagnoses

Cervical exfoliation cell specimens were collected for AutoCyte® thin-layer cytology test (TriPath Imaging Inc) and Cobas®4800 HPV assay (Roche, USA), and p16 immunocytology. The cytology results were divided into the following categories based on The Bethesda System 2014[24]: negative for intraepithelial lesions or malignancy (NILM), atypical squamous cells of undetermined significance (ASCUS), atypical squamous epithelial cells that cannot exclude high-grade lesions (ASC-H), atypical glandular cells (AGC), low grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesion (HSIL). Specimens diagnosed of ASC-H and HSIL were considered together. In an additional alternative grouping, those diagnosed as ASC-H, AGC, LSIL, AGC, ASC-H, and HSIL were considered together as ≥ LSIL.

Cobas®4800 HPV assay provides HPV16, HPV18 and pool 12 genotypes of high-risk HPV (Other HR-HPV, HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) results. For statistical analysis, HPV16 positive, HPV18 positive, and other HR-HPV positive are grouped together as HR-HPV.

For p16 immunocytology, slides were stained via an automatic immunocytochemical staining system using PathCIN® p16INK4a antibody, provided by Senying Biology, Inc. p16 cytology slides were reviewed by two senior cytologists blinded to other test results. Nuclear and cytoplasmic staining were each scored on a 0 to 3 scale, and an additive score greater than 2 of 6 was counted as positive.

Histological diagnosis of biopsy specimens was confirmed by re-examination of slides by two experienced pathologists. Diagnoses of high-grade dysplasia, including CIN2 and CIN3, were counted as positive. NILM, koilocytosis, and CIN1 were counted as negative for high-grade dysplasia. In patients who had more than one tissue sample, the worst histological diagnosis was recorded.

2.3. Statistical analysis

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for p16 immunocytology, HR-HPV testing, and liquid-based cytology was evaluated by using histological diagnoses of CIN2/CIN3 as the study endpoint. The differences in sensitivity and specificity were compared using Fisher's exact test. $P < 0.05$ was considered statistically significant.

3. Results

The characteristics of the specimens studied are summarized in Table 1. A total of 163 patients were examined, 94 from Wuhan and 69 from Shenzhen, and 116 showed atrophy. Women with atrophy were older than the population screened (51.9 *vs.* 43.4 years average). There were 116 cases positive for HR-HPV, with those from Wuhan showing a higher proportion positive than the cases from Shenzhen (84% *vs.* 52%). On routine liquid-based cytology, 103 cases were <ASCUS (NILM or koilocytic), 16 cases were ASCUS, and 44 cases were greater than ASCUS, including 1 AGC, 17 LSIL, 14 ASC-H, and 12 HSIL. On immunocytochemical analysis, 33 cases (20%) were positive for p16. Histological diagnoses included 21 cases with high-grade dysplasia (6 CIN2 and 15 CIN3), of which 13 were from cases with atrophy.

Examples of p16 immunocytology and the corresponding biopsies are shown in Fig. 1. The first is a woman of 51 years old. Her Pap smear diagnosis by routine liquid-based cytology was NILM and atrophy, but p16 immunocytology revealed a cluster of cells showing strong nuclear and cytoplasmic staining of p16 (Fig. 1A). The biopsy result was CIN3 involving endocervical glands (Fig. 1B). The second case is a 49 years old woman, whose Pap smear diagnosis was ASCUS and atrophy. Immunocytology showed some cells with nuclear and cytoplasmic p16 staining (Fig. 1C). The corresponding biopsy finding was CIN2 (Fig. 1D).

The number of cytological specimens, with and without atrophy, positive for HR-HPV, for p16, and for cytology in cases with a histological diagnosis ≤ CIN1 (i.e., false positives) and cases diagnosed as ≥ CIN2 (i.e., true positives) are detailed in Table 2. In the presence or absence of atrophy, both HR-HPV and p16 were positive for a large proportion (89–100%) of high-grade dysplasia cases. For cases without atrophy, most high-grade dysplasia cases (≥CIN2) were diagnosed as HSIL or ASC-H on cytology. For cases with atrophy, however, very few were ≥LSIL. HR-HPV testing had a high proportion of false-positives both for atrophic

Table 1
Characteristic of study populations (n).

	Total	Shenzhen, atrophy	Shenzhen, no atrophy	Wuhan, atrophy	Wuhan, no atrophy
Age ± SD	51.3 ± 4.1	52.0 ± 4.8	50.7 ± 3.7	51.9 ± 4.1	49.1 ± 2.3
HP-HPV positive	116	24	12	57	23
ASCUS	16	7	3	6	0
LSIL/AGC	18	7	5	3	3
HSIL/ASC-H	26	9	8	5	4
p16 positive	33	10	5	14	4
CIN2/CIN3	21	4	4	9	4

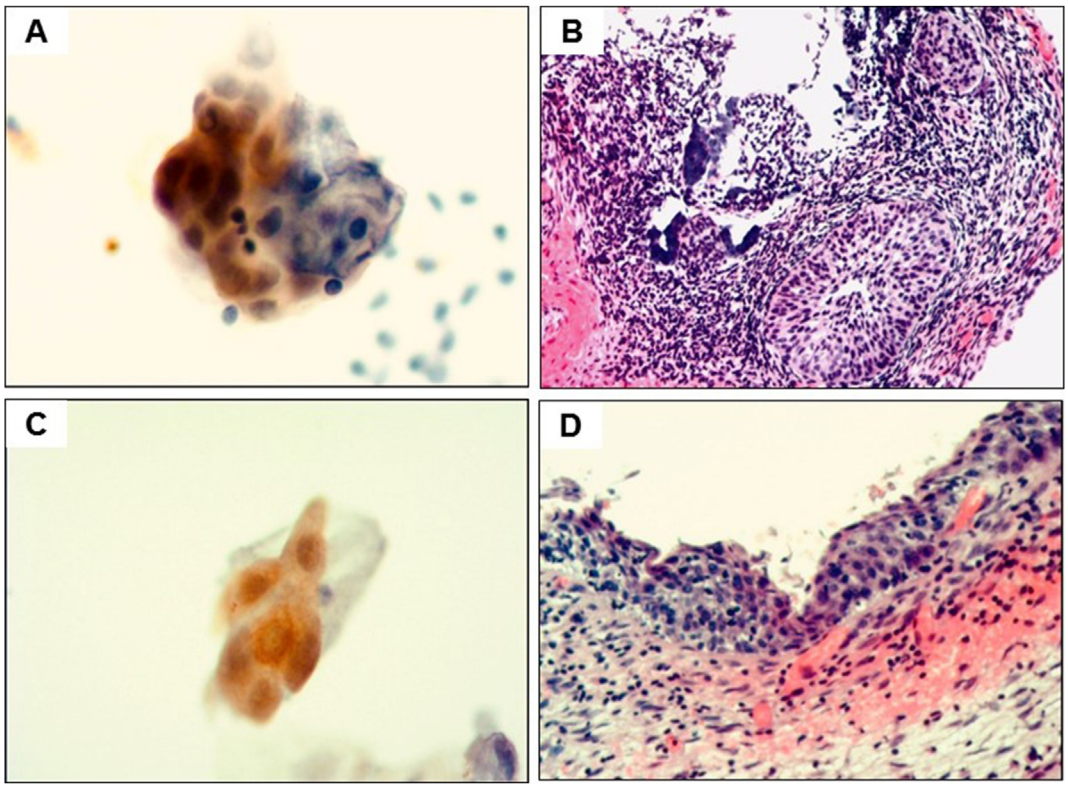

Fig. 1. p16 immunocytology and corresponding histological diagnoses.
A. Positive p16 immunochemical staining (with nuclei and cytoplasm stained brown) of cytological specimen from a 51-year-old woman diagnosed as NILM by liquid-based cytology. This case was assigned a score of 6 (3 nuclear + 3 cytoplasmic). **B**. Histological specimen corresponding to panel A, showing CIN3. **C**. Positive p16 immunochemical staining of cytological specimen from a 49-year-old woman diagnosed as ASCUS by liquid-based cytology. This case was assigned a score of 5 (3 nuclear + 2 cytoplasmic). **D**. Histological specimen corresponding to panel C, showing CIN2.

Table 2
Performance of diagnostic tests with or without atrophy n(%).

Type		n	HPV+	p16+	HSIL/ ASC-H	≥LSIL	≥ASCUS
≤CIN1	atrophy	103	69 (67)	12 (12)[a]	12 (12)	22 (21)	33 (32)
	no atrophy	39	27 (69)[a]	2 (5)[a]	6 (15)[a]	14 (37)	17 (44)
≥CIN2	atrophy	13	12 (92)	12 (92)[a]	2 (15)	2 (15)	4 (31)
	no atrophy	8	8 (100)[a]	7 (88)[a]	6 (75)[a]	6 (75)	6 (75)

[a] , $P < 0.05$, ≤CIN1 *vs.* ≥CIN2.

(67%) and non-atrophic (69%) specimens. For cases with or without atrophy, p16 immunocytochemistry had only 5%–12% false positives, and routine cytology (using a cutoff of HSIL/ASC-H) also had a low (5–13%) proportion of false-positives.

Using a histological diagnosis of high-grade dysplasia (CIN2/CIN3) as end-point, we examined the sensitivity (Fig. 2A) and specificity (Fig. 2B) of HR-HPV *vs.* p16 immunocytology *vs.* routine liquid-based cytology in atrophic cases (solid bars) or cases without atrophy (open bars). High-risk HPV had a high sensitivity for both the atrophic cases (92%) and those with no atrophy (100%). However, HR-HPV had a low specificity for detection of high-grade dyslasia for cases with (33%) or without (31%) atrophy. Routine liquid-based cytology, counting HSIL/ASC-H as positive, had a 67% sensitivity for case without atrophy, and a significantly lower sensitivity (17%) for atrophic cases. Unlike routine cytology, the sensitivity of p16 immunocytology was unaffected by the presence of atrophy. p16 immunocytology also had a high specificity for both atrophic cases and for cases without atrophy, significantly higher than HR-HPV testing. These results demonstrate that routine cytology has a low sensitivity, especially in women with atrophy, while HR-HPV testing has a high sensitivity and low specificity but p16 staining has a high sensitivity and a high specificity for cases with or without atrophy.

Combinations of the three tests examined do not perform better than p16 immunocytology alone (Table 3) and can, in some combinations

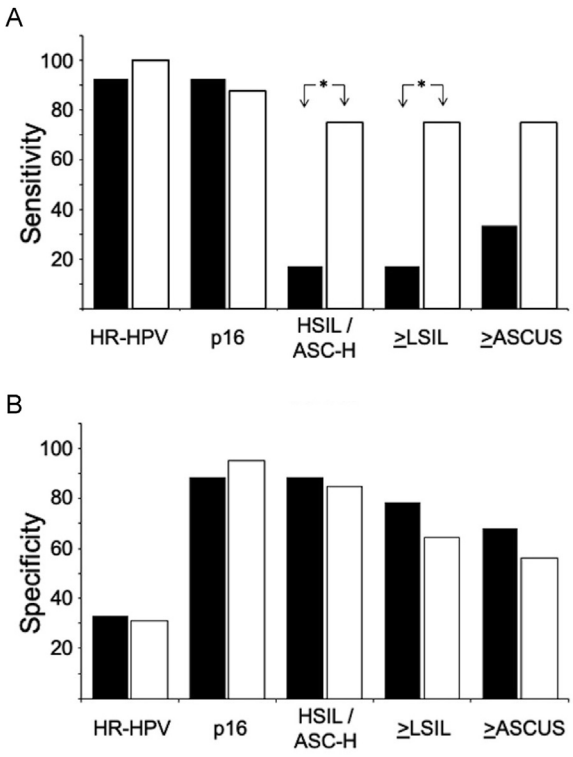

Fig. 2. Sensitivity and specificity of high-risk HPV, p16 immunocytology, and routine liquid based cytology for detection of high-grade dysplasia in specimens with and without atrophy. Solid bars, specimens with atrophy (n = 116). Open bars, specimens without atrophy (n = 47)
*, $p < 0.05$, atrophy *vs.* non-atrophy.

Table 3
Performance of diagnostic tests alone or in combination.

	n	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HR-HPV	116	95	32[a]	17[a]	98	40[a]
p16	33	90	90	58	98	90
HSIL/ASC-H	44	38[a]	87	31[a]	91[a]	81[a]
HR-HPV & p16	26	86	94	69	98	93
HSIL/ASC-H & p16	16	38[a]	98	73	91[a]	90
HR-HPV & p16 & HSIL/ASC-H	16	38[a]	98	73	91[a]	90

[a] $p < 0.05$ *vs.* p16 alone. PPV: positive predictive value; NPV: negative predictive value.

significantly decrease sensitivity and negative predictive value. For all samples (with and without atrophy), examined as a single group, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of p16 immuno-cytology are all better than or equal to HR-HPV testing or routine cytology. Combining p16 immunocytology with HPV testing and/or routine cytology had no added benefit, as compared to p16 staining alone.

4. Discussion

We find that p16 immunocytology compares favorably with routine cytology and HPV testing in the differential diagnosis of HSIL and benign atrophy. It is more sensitive than routine liquid-based cytology, particularly for atrophic specimens, and is more specific than HR-HPV testing. p16 immunocytology may decrease the need for colposcopy referrals and could be a useful tool for early detection of cervical cancer in screening peri- and post-menopausal women, who are more likely to have HSIL coexisting with atrophy.

Since wide-spread Pap screening was begun in China only in 2009, there is a large proportion of older women at first screening. Pap smears from post-menopausal women are often atrophic, which presents a diagnostic challenge.[8,10] It is difficult to appreciate HSIL in a background of atrophy because of the lack of maturation of squamous cells and similarities between atrophic cells and dysplastic cells. Highly atypical squamous cells with a high nucleus to cytoplasmic ration and a degenerative chromatin pattern that are often observed in older patients with few risk factors can elicit morphological concern for HSIL.[25] Thus, there is a particular need for improvements in differential diagnosis of benign atrophy and high-grade dysplasia in programs screening older women.

Because neither routine liquid-based cytology nor HR-HPV testing is both sensitive and specific, there is a need for a combination of tests in screening programs. HR-HPV testing is increasingly being used as a high-sensitivity, low specificity primary screening tool for prevention of cervical cancer, necessitating a triage strategy for positive cases. Besides cytology, 16/18 genotyping and evaluation of viral load have been evaluated as triage strategies.[26,27] In agreement with our results, p16 immunocytochemistry has been suggested as a useful biomarker for patients with HPV infection.[28]

There are several limitations of the present study. First, the cases studied may not represent the general screening population. The selection of cases that were referred for colposcopy was based on known HR-HPV status and/or liquid-based cytology. Thus, women negative for HR-HPV with normal no atypia are not represented. Second, it is assumed, because of the advanced age of the women in this study, that the bulk of the cases with atrophy are from post-menopausal or peri-menopausal women. However, clinical histories confirming post-menopausal status is not available. A few atrophic cases could be due to post-partum status or to contraceptive use. Finally, because a simplified scoring system is used in this study, our results may not be directly compared to previous screening studies that have used morphology-based scoring.[3,16,17,20,29]

Immunohistochemical staining of cervical biopsy specimens is in widespread use for aiding difficult diagnoses.[12] There have also been several studies that show p16 immunocytochemistry or dual staining for p16/Ki67 can aid in cytologic diagnoses.[3,16–19] Our results agree with previous studies showing the usefulness of p16 immunocytology in cervical screening studies.[21,22]

In contrast to routine liquid-based cytology, extensive expertise in cell morphology is not required for interpretation of p16 immunostained cytological specimens. Thus, it may be useful in low-resource environments and in primary hospitals. Moreover, p16 cytology can be performed at a high-throughput with a low cost and may even be adaptable to machine-based reading, suggesting it may be more useful than routine liquid-based cytology in triage of HR-HPV positive cases in large-scale cervical screening programs.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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