Original Article

p16/Ki-67 dual-stained cytology used for triage in cervical cancer opportunistic screening

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Abstract

Objective: To evaluate the efficiency of p16/Ki-67 dual stain used as a triage in cervical cancer screening.

Methods: In this study, we did 468 p16/Ki-67 dual stain in human papillomavirus (HPV) 16/18-positive or 12 other high-risk HPV (OHR-HPV) positive Thinprep cytologic test (TCT) atypical squamous cells of undetermined significance (ASCUS)/ lower-grade squamous intraepithelial lesion (LSIL) women. We evaluated the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the triage test.

Results: The sensitivity, specificity, PPV and NPV of p16/Ki-67 dual stain in HPV 16/18-positive women were 91.5%/68.4%, 77.0%/75.0%, 73.9%/59.1% and 92.8%/81.8%. In 12 OHR-HPV positive TCT ASCUS/LSIL women, the results were 79.1%/95.0%, 88.5%/66.7%, 88.5%/70.4% and 89.2%/94.1%. The risk of precancerous lesions in p16/Ki-67 dual stain positive cases was much higher than before, and the negative cases had lower risk. Besides, there was no cervical intraepithelial neoplasia (CIN) III case missed after triaged by p16/Ki-67 dual-stained cytology. In p16/Ki-67 dual-stained cytology positive women with benign pathology or CIN I, the 1-year progression rate is 20.5% and in p16/Ki-67 dual-stained cytology negative women, the 1-year progression rate is 5.6%.

Conclusions: hr-HPV genotyping test plays an important role in cervical cancer screening. p16/Ki-67 dual stain may be a promising triage test. As for chronic cervicitis or CIN I patients, a positive p16/Ki-67 dual-stained cytology suggests a high risk in progression and need to be followed up closely.

Keywords: Cervical cancer screening; hr-HPV genotyping test; p16/Ki-67 dual- stained cytology; triage test

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Introduction

Cervical cancer poses a serious threat to women's health. Many clinical studies have confirmed that persistent infection with high-risk human papillomavirus (hr-HPV) is a cause of cervical cancer and cervical intraepithelial neoplasia (CIN). HPV testing was more sensitive for the detection of grade II/III, or higher CIN or worse [CIN II or worse (CIN II+)/CIN III or worse (CIN III+)] than cytology (1,2). With the high sensitivity and negative predictive value (NPV) of HPV testing, HPV-negative women are at a very low risk for developing cervical cancer over multiple years. However, most HPV infections are harmless, and additional tests are required to identify women with progressive infections or precancerous lesions. The HPV genotype seems to be the most important factor in identifying persistent infections and disease progression. HPV 16 has the highest carcinogenic ability and may lead to 55%–60% of cervical cancer cases (1). HPV 18 is the second most common genotype and is associated with 10%–15% of cervical cancer cases (1). The remaining cases of cervical cancer are related to approximately 12 genotypes of HPV (3). Understanding the relationship between HPV and cervical cancer has led to the development of new

cervical cancer screening strategies. Presently, cotesting with HPV genotyping and cytology and HPV genotyping and reflex cytology with the atypical squamous cells of undetermined significance (ASCUS) threshold are the main screening strategies in many countries.

In updated cervical cancer screening strategies, women who test positive for HPV 16/18 are directly referred for colposcopy regardless of the cytology result. Women who test positive for hr-HPV genotypes other than 16/18 (OHR HPV) are triaged using cytology (Thinprep cytologic test, TCT); based on the HPV genotyping primary screening strategy, women with abnormal cytology findings are referred for immediate colposcopy, and those with normal cytology findings are followed up for 6–12 months (4,5). However, many HPV infections resolve spontaneously after a few months, leading to a relatively low specificity and a high referral rate for colposcopy and cervical biopsy (6). Unnecessary invasive examinations and the overtreatment of some reversible lesions may have an undesirable effect on disease progression and fertility outcomes. In addition, women who test positive for HPV may also experience anxiety. Therefore, it is of great clinical significance to explore effective triage tests to reduce colposcopy referral rate and to improve detection rate of CIN II+ patients in HPV 16/18-positive women. The optimal management of women with OHR HPV and a cytology result of ASCUS or lower-grade squamous intraepithelial lesion (LSIL) requires evaluation. The clinical management of women with cervical cancer screening results is shifting toward using risk thresholds rather than individual test results (7). Women who test positive for OHR HPV, have a cytology result of ASCUS or LSIL and have negative results in a proper triage test, could potentially avoid an immediate colposcopy referral.

Recently, p16/Ki-67 dual-stained cytology has emerged as a promising biomarker candidate. p16 is a cell-cycle regulatory protein that induces cell-cycle arrest under normal physiological conditions (8,9). The expression of a proliferation marker, such as Ki-67, within the same cervical epithelial cell may be used as a surrogate marker of cell-cycle deregulation mediated by transforming HPV infections. This morphology-independent biomarker has recently been shown to enable the efficient triage of equivocal or mildly abnormal Pap cytology results. This biomarker may be utilized as an indicator for the presence of CIN II+/III+ lesions. In this study, we evaluated the performance of p16/Ki-67 dual-stained cytology for cervical cancer screening triage in HPV 16/18-positive or OHR HPV-positive patients with ASCUS/LSIL TCT results.

Materials and methods

Study population

This study is a cross-sectional study, included an opportunistic screening population. Women who came to the Third Hospital of Peking University Gynecology Outpatient Clinic with symptoms, such as vaginal bleeding and abnormal discharge, or who simply wanted to receive a physical examination underwent HPV and cytology tests. This study was approved by Peking University Institutional Review Board and written informed consent was obtained from all patients. Patients were eligible for inclusion in the present study if they met the following criteria: 1) women between 25 and 65 years old with a positive sexual history; 2) HPV 16/18-positive or OHR HPV-positive with TCT results of ASCUS/LSIL; 3) no history of cervical HPV infection, CIN or cervical cancer; 4) no history of hysterectomy or trachelectomy; and 5) not pregnant or lactating. Many people who are HPV-positive have multiple infections. As we aimed to evaluate the performance of p16/Ki-67 dual-stained cytology for triage in HPV16/18-positive or OHR HPV-positive women with TCT results of ASCUS/LSIL, we excluded patients with multiple infections. From June 2015 to November 2016, 468 patients, which included 171 HPV 16-positive patients, 55 HPV 18-positive patients, and 242 OHR HPV-positive patients with TCT results of ASCUS/LSIL, were included the present study. According to the guidelines, HPV 16/18-positive or OHR HPV-positive patients with TCT results ≥ASCUS were referred for colposcopy and cervical biopsy. Therefore, all the patients had TCT, HPV genotyping, p16/Ki-67 dual-stained cytology, and cervical biopsy pathology results. There were no multiple infection cases in the study population. Besides, 41 OHR HPV-positive while TCT negative for intraepithelial lesions or malignancy (NILM) women underwent colposcopy.

HPV testing and cytology

The cobas® HPV test (Roche molecular diagnostics, Basel, Switzerland) was performed following the manufacturer's instructions. Pap smear samples were collected in Thinprep fixative solution, and Thinprep slides were prepared, stained, and processed with an automated stainer. All the

TCT results were confirmed by two physicians who specialized in cytology.

Cytology with p16/Ki-67 dual staining

Slides for p16/Ki-67 dual-stained cytology were prepared in the laboratory from the residual enriched cell pellet from ThinPrep specimens within two months of sample collection. A CINtec® Plus Cytology Kit (Roche mtm Laboratories AG) was used according to the instructions. Staining was performed on a Ventana Autostainer using the staining program for Thinprep slides. Samples with one or more cervical epithelial cells that simultaneously showed brownish cytoplasmic immunostaining (p16) and red nuclear immunostaining (Ki-67) were classified as positive regardless of the morphological appearance of the cells. Slides without any double-stained cells were considered negative for p16/Ki-67 dual-stained cytology. All the slides were reviewed by a trained cytologist.

Disease endpoints

All women undergoing colposcopy had at least three biopsies taken, and the majority of the patients received multiple biopsies to improve the detection of CIN. Histological evaluation was based on the CIN classification. We defined the detection of CIN II or more severe diagnoses (CIN II+) as the clinical endpoint.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), NPV and Youden's index were used to evaluate the triage value of p16/Ki-67 dual-stained cytology in cervical cancer screening. Youden's index is a single statistic that captures the performance of diagnostic tests. Youden's index = sensitivity + specificity −1. As Youden's index increases, the performance of the diagnostic test increases. The net reclassification index (NRI) is a very popular measure for evaluating the improvement in predictive performance gained by adding a marker to a set of baseline predictors. The NRI is regarded as an appropriate parameter to compare two tests. NRI = (sensitivity of test 1 – sensitivity of test 2) + (specificity of test 1 – specificity of test 2). If the NRI>0, test 1 is better than test 2. Receiver operating characteristic (ROC) curve and AUC curve were used to assess the performance of the triage tests as well. Delong's test was applied to assess the significance of difference in AUC estimates between the approaches. In addition, we followed the CIN I or chronic cervicitis patients, and compared the cumulative progression rate in the p16/Ki-67 dual-stained cytology positive and negative women. The last follow-up was September 2019. The longest follow-up time was 45 months, the shortest was 34 months and the average follow-up time was 40 months. A Kaplan-Meier curve shows the difference in terms of cumulative incidence between the p16/Ki-67 dual-stained cytology positive and negative women.

Results

Study population

Between January and November 2016, we performed p16/Ki-67 dual-stained cytology in 468 cases, among them there were 171 HPV 16-positive women, 55 HPV 18-positive women and 242 OHR HPV-positive women with TCT results of ASCUS/LSIL who underwent colposcopy. *Table 1* shows the whole distribution of the p16/Ki-67 dual-stained cytology, TCT and histopathology outcomes in women with different HPV genotypes.

In 171 HPV 16-positive women, 10 had cancer, 13 had CIN III, 48 had CIN II, and 100 had CIN less than II. The percentage of patients with CIN II+ was 41.5% (*Table 1*). Their p16/Ki-67 dual-stained cytology and cytology results are described in *Table 2*. The p16/Ki-67 dual-stained cytology-positive rates of benign neoplasia, CIN I, CIN II, CIN III and cancer were 16.7%, 23.9%, 87.5%, 100% and 100%, respectively, while the TCT positive (≥ASCUS) rates were 25.0%, 31.8%, 70.8%, 69.2% and 90.0%, respectively.

In 55 HPV 18-positive women, 3 had cancer, 16 had CIN II, and 36 had CIN less than II based on histology. The percentage of patients with CIN II+ was 34.5% (*Table 1*). Their p16/Ki-67 dual-stained cytology and cytology results are described in *Table 2*. The p16/Ki-67 dual-stained cytology-positive rates in benign neoplasia, CIN I, CIN II and cancer were 0%, 29.0%, 62.5% and 100%, respectively, while the TCT positive (≥ASCUS) rates were 20.0%, 32.3%, 56.3% and 33.3%, respectively.

In 198 OHR HPV-positive women with a TCT result of ASCUS, 1 had cancer, 6 had CIN III, 60 had CIN II, and 131 had CIN less than II based on histology. The percentage of patients with CIN II+ was 33.8% (*Table 1*). Their p16/Ki-67 dual-stained cytology results are described in *Table 3*. The p16/Ki-67 dual-stained cytology-

Table 1 Distribution of DS, TCT and histopathology outcomes in women with different HPV genotypes

Variables	n				
	Chronic cervicitis	CIN I	CIN II	CIN III	Cancer
HPV 16+					
DS+	2	21	42	13	10
DS−	10	67	6	0	0
TCT ≥ASCUS	3	28	34	9	9
TCT NILM	9	60	14	4	1
HPV 18+					
DS+	0	9	10	0	3
DS−	5	22	6	0	0
TCT ≥ASCUS	1	10	9	0	1
TCT NILM	4	21	7	0	2
OHR HPV+ TCT ASCUS					
DS+	0	15	46	6	1
DS−	15	101	14	0	0
OHR HPV+ TCT LSIL					
DS+	0	8	16	2	1
DS−	1	15	1	0	0

DS, p16/Ki-67 dual-stained cytology; TCT, Thinprep cytologic test; HPV, human papillomavirus; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesions or malignancy; LSIL, lower-grade squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia.

Table 2 Cytology with p16/Ki-67 dual staining and TCT results in HPV 16/18-positive women with different histology results

DS/TCT	n					
	Chronic cervicitis	CIN I	CIN II	CIN III	Cancer	Total
HPV 16+						
+/≥ASCUS	2/3	21/28	42/34	13/9	10/9	88
−/NILM	10/9	67/60	6/14	0/4	0/1	83
Total	12	88	48	13	10	171
HPV 18+						
+/≥ASCUS	0/1	9/10	10/9	0	3/1	22
−/NILM	5/4	22/21	6/7	0	0/2	33
Total	5	31	16	0	3	55

TCT, Thinprep cytologic test; HPV, human papillomavirus; DS, p16/Ki-67 dual-stained cytology; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesions or malignancy.

positive rates in benign neoplasia, CIN I, CIN II, CIN III and cancer were 0%, 12.9%, 76.7%, 100% and 100%, respectively.

In 44 OHR HPV-positive women with a TCT result of LSIL, 1 had cancer, 2 had CIN III, 17 had CIN II, and 24 had CIN less than II based on histology. The percentage of patients with CIN II+ was 45.5% (*Table 1*). Their p16/Ki-67 dual-stained cytology results are described in *Table 3*. The p16/Ki-67 dual-stained cytology-positive rates in benign neoplasia, CIN I, CIN II, CIN III and cancer were 0%, 34.8%, 94.1%, 100% and 100%, respectively.

Performance of p16/Ki-67 dual-stained cytology and cytology for detection of CIN II+

For the detection of CIN II+, in HPV 16-positive women,

Table 3 Cytology with p16/Ki-67 dual staining results in OHR HPV-positive women with TCT results of ASCUS/LSIL and different histology results

DS	n					
	Chronic cervicitis	CIN I	CIN II	CIN III	Cancer	Total
TCT ASCUS						
+	0	15	46	6	1	68
−	15	101	14	0	0	130
Total	15	116	60	6	1	198
TCT LSIL						
+	0	8	16	2	1	27
−	1	15	1	0	0	17
Total	1	23	17	2	1	44

OHR HPV, high-risk HPV genotypes other than 16/18; TCT, Thinprep cytologic test; ASCUS, atypical squamous cells of undetermined significance; LSIL, lower-grade squamous intraepithelial lesion; DS, p16/Ki-67 dual-stained cytology; CIN, cervical intraepithelial neoplasia.

the sensitivity, specificity, PPV, NPV and Youden's index for p16/Ki-67 dual-stained cytology were 91.5%, 77.0%, 73.9%, 92.8% and 68.5%, respectively, while the values for TCT results were 73.2%, 69.0%, 62.7%, 78.4% and 42.2%, respectively. Comparing p16/Ki-67 dual-stained cytology with TCT, the NRI was 0.263 (P<0.001) in the HPV 16-positive women. *Figure 1* shows the receiver operating characteristic (ROC) curve and area under the curve (AUC) of p16/Ki-67 dual-stained cytology *vs*. TCT in HPV 16-positive women.

In HPV 18-positive women, the sensitivity, specificity, PPV, NPV and Youden's index for p16/Ki-67 dual-stained cytology were 68.4%, 75.0%, 59.1%, 81.8% and 43.4%, respectively, while the values for TCT results were 52.6%, 69.4%, 47.6%, 73.5% and 22.0%, respectively. Comparing p16/Ki-67 dual-stained cytology with TCT, the NRI was 0.213 (P=0.137) in the HPV 18-positive women. *Figure 2* shows the ROC curve and AUC of p16/Ki-67 dual-stained cytology *vs*. TCT in HPV 18-positive women.

For the OHR HPV-positive women with TCT results of ASCUS/LSIL, sensitivity, specificity, PPV, NPV and Youden's index for the p16/Ki-67 dual-stained cytology were 79.1%/95.0%, 88.5%/66.7%, 88.5%/70.4%, 89.2%/94.1% and 67.6%/61.7%, respectively.

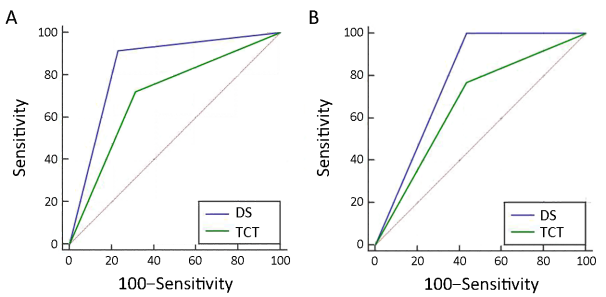

Figure 1 Receiver operating characteristic (ROC) curve of DS and TCT in HPV 16-positive women to detect CIN II or worse (CIN II+) (A) and CIN III or worse (CIN III+) (B). (A) The AUC of DS and TCT is 0.842 and 0.705. Difference between areas is 0.138, 95% CI is 0.062 to 0.213; (B) The AUC of DS and TCT is 0.783 and 0.667. Difference between areas is 0.115, 95% CI is 0.022 to 0.208. DS, p16/Ki-67 dual-stained cytology; TCT, Thinprep cytologic test; HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; AUC, area under the curve; SE, standard error; 95% CI, 95% confidence interval.

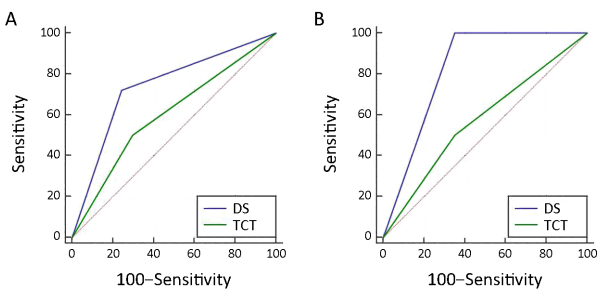

Figure 2 Receiver operating characteristic (ROC) curve of DS and TCT in HPV 18-positive women to detect CIN II or worse (CIN II+) (A) and CIN III or worse (CIN III+) (B). (A) The AUC of DS and TCT is 0.739 and 0.601. Difference between areas is 0.138, 95% CI is −0.051 to 0.327; (B) The AUC of DS and TCT is 0.824 and 0.574. Difference between areas is 0.250, 95% CI is −0.045 to 0.545. DS, p16/Ki-67 dual-stained cytology; TCT, Thinprep cytologic test; HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; AUC, area under the curve; SE, standard error; 95% CI, 95% confidence interval.

Triage of HPV 16/18-positive or OHR HPV-positive women with TCT results of ASCUS/LSIL

We evaluated the performance of p16/Ki-67 dual-stained cytology for triaging women who were HPV 16/18-positive or OHR HPV-positive with TCT results of ASCUS/LSIL. In HPV 16-positive women, the colposcopy referral rate was reduced to 48.5% (83/171) if the triage test was negative, among whom 7.2% (6/83) had CIN II. In HPV 18-positive women, 60.0% (33/55) of women avoided colposcopies, but 18.2% (6/33) of these women had CIN II. In OHR HPV-positive women with TCT results of ASCUS/LSIL, 60.7% (147/242) of these women avoided immediate colposcopy, 10.2% (15/147) of whom had CIN II. Fortunately, no CIN III or cancer cases were missed after triage tests in the study population.

Risk stratification of p16/Ki-67 dual-stained cytology

We compared the risk of precancerous lesions in women who were HPV 16/18-positive or OHR HPV-positive with TCT results of ASCUS/LSIL. The risk for CIN II+ in p16/Ki-67 dual-stained cytology-negative patients was much lower than that in p16/Ki-67 dual-stained cytology-positive patients. We simultaneously compared the value of p16/Ki-67 dual-stained cytology and TCT for triage (*Figures 3–6*).

Cytology p16/Ki-67 dual staining results in CIN II+ women with a TCT result of NILM

In the HPV 16-positive women, there were 48 CIN II patients, 13 CIN III patients, and 10 cancer patients, among whom 14 patients, 4 patients, and 1 patient, respectively, had a TCT result of NILM. For the HPV 18-positive women, there were 16 CIN II patients and 3 cancer patients, among whom 7 and 2 patients, respectively, had a TCT result of NILM. The HPV 16/18-positive patients with a TCT result of NILM and pathological findings of CIN II+ were missed when screening by TCT. Thus, we analyzed these patients as a special population.

The positivity rate of p16/Ki-67 dual-stained cytology in HPV 16-positive patients with a TCT result of NILM and pathological findings of CIN II, CIN III and cancer were 78.6% (11/14), 100% (4/4), and 100% (1/1), respectively.

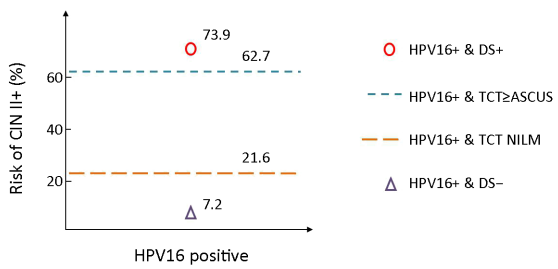

Figure 3 Risk for cervical precancerous lesions according to DS and cytology results in HPV 16-positive women, among whom the risk was originally 41.5%. CIN, cervical intraepithelial neoplasia; CIN II+, CIN II or worse; HPV, human papillomavirus; DS, p16/Ki-67 dual-stained cytology; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesions or malignancy.

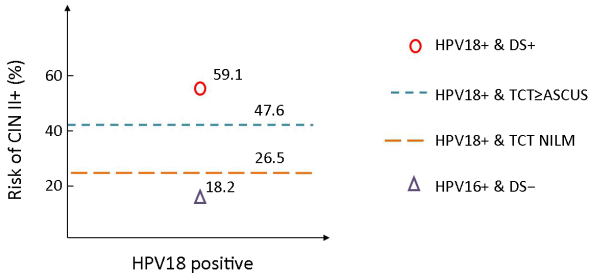

Figure 4 Risk for cervical precancerous lesions according to DS and cytology results in HPV 18-positive women, among whom the risk was originally 34.5%. CIN, cervical intraepithelial neoplasia; CIN II+, CIN II or worse; HPV, human papillomavirus; DS, p16/Ki-67 dual-stained cytology; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesions or malignancy.

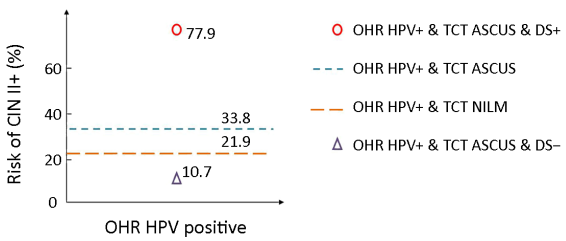

Figure 5 Risk for cervical precancerous lesions according to DS results in OHR HPV-positive women with a TCT result of ASCUS. The risk for OHR HPV-positive women with a TCT result of NILM was 21.9% according to the data from 41 OHR HPV-positive women with a TCT result of NILM who underwent colposcopy. CIN, cervical intraepithelial neoplasia; CIN II+, CIN II or worse; HPV, human papillomavirus; DS, p16/Ki-67 dual-stained cytology; OHR HPV, high-risk HPV genotypes other than 16/18; TCT, Thinprep cytologic test; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraepithelial lesions or malignancy.

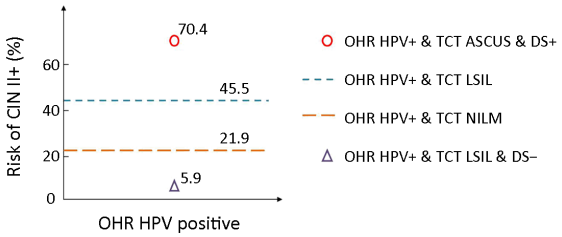

Figure 6 Risk for cervical precancerous lesions according to DS results in OHR HPV-positive women with a TCT result of LSIL. The risk for OHR HPV-positive women with a TCT result of NILM was 21.9% according to the data from 41 OHR HPV-positive women with a TCT result of NILM who underwent colposcopy. CIN, cervical intraepithelial neoplasia; CIN II+, CIN II or worse; HPV, human papillomavirus; DS, p16/Ki-67 dual-stained cytology; OHR HPV, high-risk HPV genotypes other than 16/18; TCT, Thinprep cytologic test; LSIL, lower-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesions or malignancy.

In HPV 18-positive patients with a TCT result of NILM and pathological findings of CIN II and cancer, the positivity rate of p16/Ki-67 dual-stained cytology was 71.4% (5/7) and 100% (2/2), respectively.

Predictive value of p16/Ki-67 dual-stained cytology

There were 291 patients with CIN<II. Among these patients, 55 were p16/Ki-67 dual-stained cytology-positive, and 236 were p16/Ki-67 dual-stained cytology-negative.

In the p16/Ki-67 dual-stained cytology-positive women, 11 patients progressed to CIN II, and 11 patients were lost to follow-up. These 11 patients progressed in 3 months, 4 months, 5 months, 6 months, 7 months, 7 months, 7 months, 12 months, 12 months, 15 months and 16 months, respectively. The cumulative progression rate at 1 year was 20.5% (9/44).

For the p16/Ki-67 dual-stained cytology-negative women, 16 patients progressed to CIN II, and 40 patients were lost to follow-up. Among these 16 patients, 1 patient progressed in 5 months; 2 patients progressed in 6 months; 1 patient progressed in 7 months; 1 patient progressed in 8 months; 1 patient progressed in 9 months; 3 patients progressed in 10 months; 1 patient progressed in 11 months; 1 patient progressed in 12 months; 1 patient progressed in 14 months; 1 patient progressed in 21 months; 1 patient progressed in 23 months; 1 patient progressed in 25 months and 1 patient progressed in 30 months. The cumulative progression rate at 1 year was 5.6% (11/196), and the 2-year progression rate was 7.1% (14/196).

The total cumulative progression rate and risk in the p16/Ki-67 dual-stained cytology-positive women was significantly higher than that in the p16/Ki-67 dual-stained cytology-negative women (25.0% *vs.* 8.2%, P=0.0110). *Figure 7* shows the difference in terms of cumulative incidence between the p16/Ki-67 dual-stained cytology positive and negative women.

Discussion

Cervical cancer is one of the most common malignant tumors in China. With a definitive understanding of the cause and development of this disease, proper screening may reduce the risk of death. Cervical cancer screening based on primary HPV testing, alone or in conjunction with cytology, has been successfully evaluated in clinical trials. However, many deficiencies still exist in the current screening techniques and strategies; for example, HPV testing has twice as many positive results as cytology-based screening (10). With advancements in cervical cancer screening research, immunochemical staining in cervical cytology has shown great promise in early screening for cervical cancer, especially for triage in cervical cancer screening (11,12).

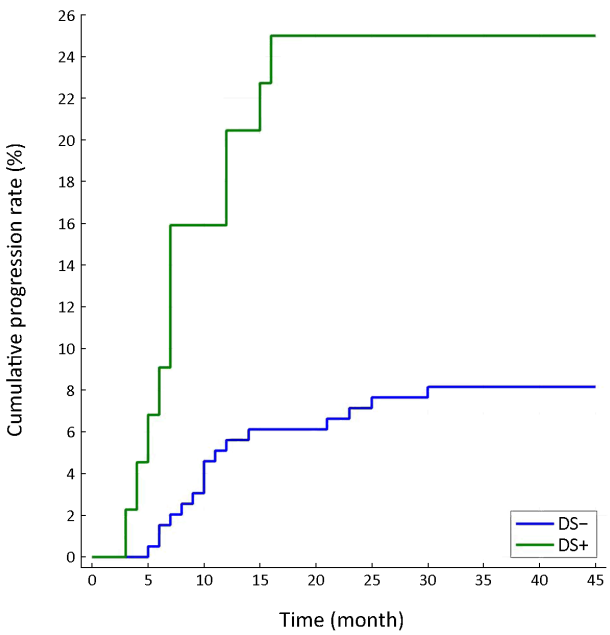

Figure 7 Difference in terms of cumulative incidence between p16/Ki-67 dual-stained cytology positive (DS+) and negative (DS−) women. Chi-squared is 6.4659, P=0.0110.

A previous study showed that p16/Ki-67 dual-stained cytology had a high sensitivity and moderate specificity for detecting underlying cervical precancerous lesions and cancers in various settings (13). We evaluated the clinical performance of p16/Ki-67 dual-stained cytology in women undergoing contesting with HPV genotyping and cytology. Petry *et al*. were the first to examine the use of p16/Ki-67 dual-stained cytology in HPV-positive women with normal cytology and reported that dual-stained cytology performed well for the detection of women who were at risk for developing high-grade CIN lesions (14). Uijterwaal *et al*. compared p16/Ki-67 dual-stained cytology and HPV tests for triage in a population with a TCT result of ASCUS. Dual-stained cytology had a relatively higher specificity and maintained good sensitivity (15). Another study evaluated p16/Ki-67 dual-stained cytology for triage in an HPV-positive population with a TCT result of NILM. The HPV genotyping test had a lower sensitivity than dual-stained cytology (16). Several studies have focused on the value of p16/Ki-67 dual-stained cytology used as a triage test (17); A study evaluated p16/Ki-67 dual-stained cytology in the triage of women with ASCUS by comparing this method with an HPV DNA assay, and the results showed that this dual staining method may be a promising tool in the triage of ASCUS (12), yet no similar target population has been found so far.

In HPV 16-positive women, nearly 50% of patients' colposcopy biopsy results are negative (18). The specificity of screening is relatively low, and unnecessary colposcopy has a certain impact. Using a triage test to improve the detection rate of CIN II+ may help to reduce the referral rate for colposcopy in the HPV 16-positive women. Our results showed that for triaging these women, p16/Ki-67 dual-stained cytology had a specificity and an NPV of 77.0% and 92.8%, respectively, while maintaining a relatively high sensitivity and PPV of 91.5% and 73.9%, respectively. The colposcopy referral rate was reduced by 48.5% after triage testing, while p16/Ki-67 dual-stained cytology-positive women had a relatively high risk for developing CIN II+. The incidence of CIN II+ in dual-stained cytology-negative patients was only 7.2% in the HPV 16-positive women. Compared with TCT, the NRI of p16/Ki-67 dual-stained cytology was 0.263 ($P<0.001$). Thus, dual-stained cytology has a better triage value than TCT.

For HPV 18-positive women, nearly 70% of whom had negative colposcopy biopsy results if they were directly referred (18). In our study, the risk for developing CIN II+ in HPV 18-positive women was 34.5%, and the risk increased to 59.1% if p16/Ki-67 dual-stained cytology was positive. If dual-stained cytology was negative, the risk for developing CIN II+ decreased to 18.2%. The colposcopy referral rate was reduced by 60.0% after triage testing, with a CIN II+ proportion of 18.2% in dual-stained cytology-negative patients. These results show that this method has a relatively satisfactory triage value. Compared with TCT, the NRI of p16/Ki-67 dual-stained cytology was 0.213 ($P=0.137$) in HPV 18-positive women. The accuracy of cytology is highly dependent on the experience of the physician specializing in cytology. The interpretation standards of p16/Ki-67 dual-stained cytology are more concise and easier to interpret than traditional cytology, which is a great advantage in developing countries.

According to the guidelines (19), OHR HPV-positive women need to undergo cytology testing, and TCT results of ≥ASCUS should be referred for colposcopy. Simms *et al*. compared the risk of cervical cancer over 20 cumulative years with the direct referral for colposcopy, and after 12 months, the OHR HPV-positive women with TCT results of ASCUS/LSIL had risks that were low and not significantly different (1.0% *vs*. 1.2%). However, referral for colposcopy significantly increased the cost of screening. The risk of CIN II+ within 1 year for OHR HPV-positive women with TCT results of ASCUS/LSIL was 6.0%−11.0% (20). Whether or not OHR HPV-positive women with TCT results of ASCUS/LSIL need to immediately undergo colposcopy is still debated. In our study, we used p16/Ki-67 dual-stained cytology, which has an ideal sensitivity and specificity, as a triage test. The risk of developing CIN II+ increased to 77.9%/70.4% after triage testing, while p16/Ki-67 dual-stained cytology-positive and OHR HPV-positive women with TCT results of ASCUS/LSIL may have had a relatively higher risk for developing CIN II+. The risk of developing CIN II+ in dual-stained cytology-negative patients was 10.7%/5.9%, which was far lower than that in the OHR HPV-positive women with a TCT result of NILM who were not referred for colposcopy according to the guidelines. No CIN III patients were missed after triage by p16/Ki-67 dual-stained cytology, suggesting that p16/Ki-67 dual-stained cytology may be a promising triage test.

In addition, there were patients who were HPV-positive with a TCT result of NILM and developed CIN II+ in our study; these patients would have been missed if they had been screened by cytology alone. In HPV 16-positive women with a TCT result of NILM, there were 14 CIN II

patients, 4 CIN III patients, and 1 cancer patients. In HPV 18-positive women with a TCT result of NILM, there were 7 CIN II patients and 2 cancer patients. In our study, the positivity rate for p16/Ki-67 dual-stained cytology was 100% in HPV-positive patients with a TCT result of NILM who developed CIN III or cancer, suggesting that dual-stained cytology is much more sensitive than TCT. Petry *et al*. evaluated p16/Ki-67 dual-stained cytology for triaging HPV-positive patients with a TCT result of NILM and found a sensitivity and specificity of 91.9% and 82.1%, respectively, which was similar to the results (14). In addition, all the CIN III or cancer patients had a positive result in p16/Ki-67 dual-stained cytology, suggesting that there was an ideal NPV. In our study, the incidence of CIN II+ in OHR HPV-positive patients with a TCT result of ASCUS and p16/Ki-67 dual-stained cytology-positive results (77.9%) was higher than the incidence of 73.9% among HPV 16-positive and p16/Ki-67 dual-stained cytology-positive patients, 59.1% for HPV 18-positive and p16/Ki-67 dual-stained cytology-positive patients, and 70.4% for OHR HPV-positive patients with a TCT result of LSIL and p16/Ki-67 dual-stained cytology-positive results. Differences in the study population may have caused this result.

Literature reported that cumulative 3-year progression rate of women with normal colposcopy cervical biopsy results to CIN II+ and CIN III+ is 1.86% and 0.64%, respectively (21). Ten percent of women with colposcopy cervical biopsy results of CIN I will progress to CIN II within 2 years (22). Regular follow-up increase patients' cost and can be easily missed for women with normal colposcopy cervical biopsy results or CIN I. Therefore, predicting the progression of the disease would greatly benefit follow-up management and patient education. In our study, the cumulative progression rate of p16/Ki-67 dual-stained cytology positive women is significantly higher than the negative women. Our result shows the cumulative 1-year progression rate was 20.5%, suggesting a high short-term risk. From the point of detecting principle view, p16 and Ki-67 expressing in the same cell highly suggests the disorder of cell cycle, even the cervical biopsy results are normal, which also strongly indicates that the case has higher risk of progressing to CIN II+ and should be followed up more rigorously.

As this is an exploratory study and we aimed to evaluate the performance of p16/Ki-67 dual-stained cytology for triage in specific population, we excluded patients with multiple infections, but we cannot deny that the result maybe has selection bias. The results still show that p16/Ki-67 dual-stained cytology is useful for triaging HPV 16/18-positive patients or OHR HPV-positive patients with TCT results of ASCUS/LSIL. This method may be a promising triage test for reducing colposcopy referrals while maintaining high sensitivity for detecting cervical precancerous lesions. For chronic cervicitis or CIN I patients, positive p16/Ki-67 dual-stained cytology results suggest a high risk for progression, which need to be closely followed up.

Conclusions

Cervical cancer is a threaten to women all over the world and cervical cancer screening may reduce the morbidity and mortality by detecting cervical intraepithelial neoplasia. hr-HPV genotyping test plays an important role in cervical cancer screening. p16/Ki-67 dual stain may be a promising triage test. As for CIN I or chronic cervicitis patients, a positive p16/Ki-67 dual-stained cytology suggests a high risk in progression and need to be followed up closely.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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