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Evaluation of p16/Ki67 dual staining compared with high-risk HPV testing to assess liquid-based cytology with atypical squamous cells of unknown significance

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Summary

Objective: To compare the clinical performance of p16/Ki67 dual staining and high risk-human papilloma virus (HR-HPV) testing as an auxiliary monitoring index to triage atypical squamous cells of undetermined significance (ASCUS). *Materials and Methods:* Fifty-four patients diagnosed with ASCUS by liquid-based cytology were inspected by colposcopy-guided biopsy and cervical histopathological examination. The cytological samples were tested for HR-HPV with Cervista HPV assays, and the cell morphology was evaluated with dual staining using the CINtec PLUS kit. ROC curves were used to evaluate the diagnostic value of p16/Ki67 dual staining and HR-HPV assays to detect underlying CIN2+ in ASCUS patients. The kappa value assessed the reliability of p16/Ki67 dual staining and HR-HPV, and the diagnostic advantages of the two tests were analyzed using logistic regression analysis. *Results:* Twenty-two of the 54 ASCUS cases were diagnosed as CIN2+ by histopathology. The area under the ROC curve was 0.776 (95%CI 0.647-0.904) and 0.689 (95%CI 0.548-0.830) for CIN2+ detection using p16/Ki67 dual-staining cytology and HR-HPV, respectively. P16/Ki67 dual staining demonstrated a sensitivity and negative predictive value similar to that of HR-HPV for detecting underlying CIN2+ in ASCUS (86.36% *vs.* 90.91%, 88.00% *vs.* 88.24%). There was a certain degree of concordance between the p16/Ki67 dual staining cytological assay and the HR-HPV test (kappa = 0.315, $p = 0.015$). The accuracy of CIN2+ diagnosis using p16/Ki67 dual staining cytology was higher than that of HR-HPV (OR=11.025 *vs*. OR=6.026, $p = 0.001$). *Conclusions:* P16/Ki67 dual staining and HR-HPV can be used as an auxiliary monitoring index to triage ASCUS. The diagnostic value of p16/Ki67 dual staining was superior to that of HR-HPV for detecting underlying CIN2+ in ASCUS.

Key words: Human papillomavirus (HPV); Atypical squamous cell of undetermined significance(ASCUS); P16/Ki67 dual staining; Cervical intraepithelial neoplasia (CIN).

Introduction

The Bethesda system (TBS) classification of cervical cells has been accepted and applied in many countries and regions, although the assessment of atypical squamous cells of unknown significance (ASCUS) remains a difficult problem for clinicians. ASCUS might be a response to reactive changes or may mask an additional lesion. Jones suggested that ASCUS account for 5~l0% of high-grade cervical lesions and 0.1% of invasive cervical cancers [1]. If patients with ASCUS received only a follow-up, some of these patients would lose their best opportunity for treatment. However, if all of the patients were inspected by colposcopy and cervical biopsy, then many patients with benign lesions would receive unnecessary procedures, resulting in the waste of medical resources.

The definite recognition of HPV as the etiological agent of cervical cancer led to its adoption as a cervical cancer screening strategy [2]. Currently, high-risk human papillomavirus (HR-HPV) detection has been widely used to triage patients with ASCUS, and it plays an important role in the diagnosis and treatment of cervical lesions in ASCUS [3, 4]. HPV [1] testing as a cervical cancer screening strategy has been evaluated and exhibits sensitivity in identifying high-grade cervical intraepithelial neoplasia (CIN) [5]. However, the main disadvantage of cytology/HPV co-testing is the limited specificity for CIN2+ [6]. HPV testing has been found to be mostly inefficient because the majority of ASCUS cases are infected with HR-HPV types [7]. Moreover, HPV testing cannot distinguish patients with transient infections from those with persistent infections, although the former can be cleared and does not cause cervical lesions [8]. Therefore, alternative monitoring strategies are urgently needed.

Dual staining for p16/Ki67 has been used to increase interobserver agreement and stratify patients for treatment purposes [9]. After staining, the p16-stained cytoplasm is brownish yellow, and the Ki67-positive nucleus is red. Ki67 is a proliferation-associated antigen, and the cyclin-dependent kinase inhibitor p16 functions as a tumor suppressor in

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cell cycle control. Under normal physiological conditions, these two mutually exclusive proteins are not likely to be co-expressed in the same cervical cells. Thus, the expression of p16 and Ki67 in the same cervical cell suggests Rb inactivation and abnormal cell cycle regulation [10]. Dual staining simultaneously detects the over-expression of both p16 and Ki67 after persistent HR-HPV infection. In fact, p16/Ki67 coexpression is a sign of HR-HPV-induced cell cycle deregulation and indicates a transforming HR-HPV infection and the presence of high-grade CIN lesions [11, 12]. This phenomenon is independent of morphology and provides an objective indicator to detect underlying high-grade lesions. Positive p16/Ki67 dual staining suggests CIN2+ lesions, and the patient should receive a referral for a colposcopy. In contrast, patients with negative p16/Ki67 dual staining should be followed-up after one year. Previous studies on p16/Ki67 dual staining have focused on the triage of HPV-positive women [11, 13, 14] or a colposcopy referral population [15].

The purpose of this study was to detect p16/Ki67 dual staining using immunocytochemistry and HR-HPV testing using Cervista HPV assays in ASCUS patients with underlying cervical HR-CIN 2/3 and to explore the clinical value of these two tests as auxiliary monitoring indices in the triage of patients with ASCUS.

Materials and Methods

The prospective study involved 1310 women referred to in- and out-patient clinics in the Department of Gynecology at the Second Affiliated Hospital, Jiaxing College Medical School, China, from May to July 2016. All 65 samples were diagnosed with ASCUS, and 54 women were evaluated using colposcopy-guided biopsy. The average age of the women was 45.63 ± 11.90 (range 26 to 72) years. Samples from the women were collected using liquid-based cytology (LBC) and HR-HPV testing at their first clinical visit. In addition, dual staining was performed on the remaining LBC. The study was approved by the ethics committee of the hospital, and all of the participants provided informed consent.

Cervical samples were collected using a cervical brush, and the samples were subsequently transferred to PreservCyt solution. Thin layer sliders were prepared using a ThinPrep 2000 slide processor. Two trained cytopathologists independently analyzed the cervical cytology samples and classified the tissues according to the Bethesda 2001 guidelines. The tissue samples were disposed of according to standard histological procedures and classified in accordance with the 2002 WHO guidelines for cervical histopathology. Two experienced pathologists interpreted all of the histological slides. According to pathological diagnosis, histological diagnoses were divided into chronic inflammation, CIN1, CIN2, and CIN3. According to the 2001 American Society for Colposcopy and Cervical Pathology (ASCCP) jointly developed guidelines for the management of cervical precancerous lesions, CIN1 can be followed-up, but CIN2 and CIN3 are associated with a HR and should be controlled by clinical prevention and appropriate treatment. In this study, negative (including inflammation, reactive alterations and squamous metaplasia) and CIN1 cases were referred to as the low-grade lesion group, and CIN2/CIN3 cases were referred to as the high-grade lesion group.

Cervista HR-HPV has been approved by the US Food and Drug Administration (FDA) and is a new technology [16]. The detection of Cervista HR-HPV was performed according to the manufacturer's instructions, and the HR-HPV assay is an FDA-approved qualitative diagnostic test. This method can detect 14 types of HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) based on the PCR-amplification of HR-HPV-DNA followed by nucleic acid hybridization. The Cervista HR-HPV test was performed according to the manufacturer's instructions. The HR-HPV DNA samples were extracted from residual cervical samples using a DNA extraction jit and tested using Cervista HR-HPV.

New slides from the residual LBC samples in ThinPrep Papanicolaou (Pap) test LBC vials were prepared using the ThinPrep 2000 slide processor. The cytology kit is an immunocytochemistry assay for the simultaneous qualitative detection of p16 and Ki67 proteins in cervical cytology preparations. Dual staining was performed according to the manufacturer's instructions. A two-step immunocytochemical staining procedure was performed on cervical cytology preparations. The kit contains a ready-to-use primary antibody cocktail containing a mouse monoclonal antibody (clone E6H4) against human p16 protein and a rabbit monoclonal antibody (clone 274-11 AC3) against human Ki67. Two experienced cytotechnologists blinded to the results of the cytological and histological diagnoses independently analyzed and evaluated the double-immunostained slides. The presence of one or more cervical epithelial cells showing brown cytoplasmic immunostaining and red nuclear immunostaining within the same cell was regarded as a positive CINtec PLUS cytology test result (brown cytoplasmic staining for p16 and red nuclear staining for Ki67); otherwise, the test results were considered negative.\

Using CIN2+ as the gold standard, receiver-operating characteristic curves (ROCs) of dual staining and HR-HPV were drawn to evaluate the diagnostic value of the two indices. To examine the reliability of dual staining, HR-HPV and histopathology, the kappa statistic was used. Kappa values between 0.40 and 0.60 were considered moderate and those between 0.6-0.8 were considered robust. The OR and 95% confidence intervals (95% CI) were assessed using conditional logistic regression for different clinical trials. $P < 0.05$ was considered statistically significant.

Results

From 1,310 LBC specimens categorized as ASCUS (n=65) and 11 cases were excluded due to incomplete data (no results from dual-staining or cervical biopsy). Thus, a total of 54 ASCUS samples were available for the study cohort. Among these samples, 22 cases (40.74%) were confirmed as CIN2/3 by colposcopy and cervical biopsy. No cases of cervical cancer were detected. Table 1 shows the distribution of the four pathological diagnoses (negative for dysplasia, CIN1, CIN2, and CIN3) in the 54 ASCUS cases.

ROC curves were fitted using dual staining and HR-HPV as test variables with histopathology as a gold standard. Table 2 and Figure 1 show that when p16/Ki67 dual staining was used as the test variable, the AUC was 0.776 (95% CI 0.647-0.904), and the sensitivity, specificity, and positive and negative predictive values for p16/Ki67 dual staining was 86.36%, 68.75%, 65.52%, 88.00%, respectively. When HR-HPV was used as the test variable, the AUC was 0.689 (0.548-0.830), and the sensitivity, specificity, and

Table 1. — *Correspondence between ASCUS and histopathology in 54 cases.*

Cytology	Biopsy			
	Negative	CIN 1	CIN 2	CIN3
ASCUS (n=54)	20	12	14	8

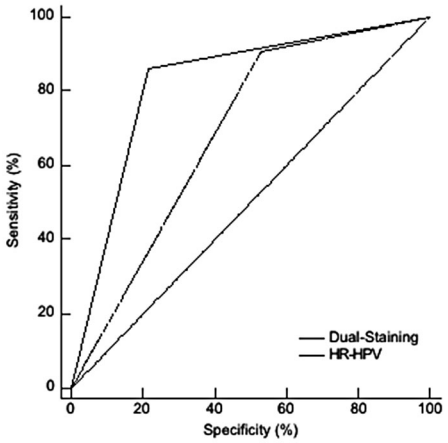

Figure 1. — ROC analysis of p16/Ki67 dual staining and HR-HPV detection of underlying CIN2+ in ASCUS.

Table 2. — *ROC analysis of p16/Ki67 dual staining and HR-HPV for the detection of underlying CIN2+ in ASCUS.*

Variables	AUC	SE	95% CI	*p*
Dual staining	0.776	0.066	0.647-0.904	0.001
HR-HPV	0.689	0.072	0.548-0.830	0.019

Table 3. — *Consistency Test for P16/Ki67 dual-staining and HR-HPV.*

	HR-HPV		Kappa	*p*
	Positivity	Negative		
Dual staining			0.315	0.015
Positivity	23	5		
Negative	14	12		

Table 4. — *Logistic regression analysis of the degree of coincidence between p16/Ki67 dual staining and HR-HPV for CIN2+ diagnosis*

Variable	B	Wald	P	OR	95% CI
Dual staining	2.400	10.039	0.002	11.025	2.498-48.662
HR-HPV	1.796	3.986	0.046	6.026	1.033-35.137
Constant	-3.213	10.903	0.001		

positive and negative predictive values for HR-HPV was 90.91%, 46.88%, 54.05%, 88.24%, respectively. Both of them had significant diagnostic value ($p < 0.05$). These two methods can be used as diagnostic indices for CIN2+.

Using histopathology as the gold standard compared with the other two methods, the consistency test of the diagnostic results for dual staining, HR-HPV and CIN2+ was evaluated using the kappa coefficient. The consistency of these indicators was statistically significant ($p < 0.05$). Dual-staining cytology was superior to HR-HPV for CIN2+ diagnosis (0.525 *vs.* 0.341). The consistency test of the diagnostic results for dual staining and HR-HPV was further analyzed, and the results, shown in Table 3, revealed the degree of diagnostic consistency between the two tests ($p = 0.015$).

Using the CIN2+ results as the dependent variable and dual staining and HR-HPV results as independent variables, binary logistic regression analysis was used for further analysis. As shown in Table 4, the indicators for the two methods were statistically significant ($p < 0.05$). With reference to the OR value, dual staining is superior to HR-HPV for diagnosing CIN2+ (11.025 *vs*. 6.026).

Discussion

p16 and Ki67 have been widely recognized as markers for the diagnosis of cervical lesions. In previous studies, p16 and Ki67 have been detected by immunohistochemistry [17-19] in samples obtained from cervical biopsy, resulting in some trauma to patients, and a certain proportion of false negative and false positive tests. Compared with histopathological examination, cytological testing uses residual cervical liquid-based non-invasive cytology samples without repeated sampling. In addition, p16/Ki67 dual staining can be conducted without respect to age [20]. p16/Ki67 dual staining is an independent method to evaluate the morphological changes to cells and plays an auxiliary role in the morphological diagnosis of difficult ASCUS cases, such as crowded HSIL cell groups or reactive changes. p16/Ki67 dual staining can facilitate improvements in the level of screening for cell-based cervical cancer.

The purpose of cervical cancer screening is to identify CIN2+ lesions. As high-grade CIN have a substantial risk in developing cervical carcinoma, the screening strategy should have high sensitivity. HPV testing is known to significantly increase the sensitivity for the detection of CIN2+ or CIN3+ when used as an adjunctive test to Pap cytology based screening or as a primary screening method [21, 22]. High specificity can prevent the negative side effects of patient overtreatment. Optimum screening improves the sensitivity of cytology without reducing the specificity or masking high-grade lesions and uses biomarkers to assist in cervical smear interpretation and diagnosis. The aim of this study was to evaluate the clinical performance of p16/Ki67 dual staining and HR-HPV to triage ASCUS patients for high-grade CIN detection, and CIN2+ was the end point. The results showed p16/Ki67

dual-staining and HR-HPV can be used as an auxiliary monitoring index to triage ASCUS, and there was a certain degree of consistency between the two methods.

The ROC curve is a comprehensive, effective diagnostic tool linking the sensitivity and specificity of diagnostic tests. The area under the ROC curve (AUC) is generally accepted as an indicator evaluating the validity of diagnostic tests. The diagnostic value of AUC is low at 0.5~0.7, moderate at 0.7~0.9, and high when values are greater than 0.9. In this study, the results of ROC curve analysis showed that the AUC of p16/Ki67 dual staining was 0.776, a moderate level. However, the AUC of HR-HPV was 0.689, which is a low level (Table 2 and Figure 1). This finding indicated that the two methods could be used as diagnostic indices for CIN2+ ($p < 0.05$). It indicated not only that HPV can be used as the diagnostic tool, but also P16/ki67 dual-staining can detect underlying CIN2+ in women with ASCUS. A similar effect has been observed in the recent studies [11, 20].

The sensitivity, specificity, and positive and negative predictive values of HR-HPV testing were 90.91%, 46.88%, 54.05%, and 88.24%, respectively, for CIN2+ detection. The sensitivity and negative predictive values were satisfactory, but the specificity and positive predictive value of HR-HPV testing were not robust enough. It has been suggested that the combination of HR-HPV testing and cytological screening increases the sensitivity of high-grade lesion detection but reduces detection specificity [6, 23, 24]. In this study, the specificity of p16/Ki67 dual staining was 68.75% for detecting CIN2+, and the PPV was 65.52%; both values were higher than those of HR-HPV, although comparative sensitivity and negative predictive values were observed between the two tests. This finding indicated that p16/Ki67 dual staining not only shows better sensitivity and negative predictive values than HR-HPV, but also compensates for deficiencies in specificity and PPV. The results of this study are consistent with the data from previous studies showing that p16/Ki67 dual-staining cytology has high sensitivity and specificity for CIN detection [15, 20]. The results of this study were lower than those of Schmidt *et al.* [20], who reported that the sensitivity of p16/Ki67 dual staining for CIN2+ in patients with ASCUS was 92.2% and the specificity was 80.6%. One reason for this discrepancy was related to the skill of technologists, another was the different standards of the research objects. The increased specificity and PPV of dual staining indicated that the number of patients referred for colposcopy might decrease, thereby saving medical expenses. Therefore, p16/Ki67 dual-staining greatly improved the specificity and positive predictive value of identifying underlying CIN2+ in ASCUS patients.

In this study, the consistency test revealed a degree of consistency between p16/Ki67 dual staining and HR-HPV, suggesting that both tests can detect underlying CIN2+ in ASCUS (Table 3). Waldstrom *et al.* reported the concordance of p16/Ki67 dual-staining and HPV was 69.5% (kappa=0.361) [25]. Further examination of the diagnostic advantages of both tests using logistic regression analysis showed that the diagnostic OR of p16/Ki67 dual staining was better than that of HR-HPV (11.025 *vs*, 6.026). Based on the OR, p16/Ki67 dual staining was more closely related to CIN2+, thus the diagnostic advantage of p16/Ki67 dual staining was better than that of HR-HPV. It has been suggested that dual staining is more accurate for identifying underlying CIN2+ in ASCUS. The results of this study confirmed previous findings, which suggested that p16 and Ki67 expression might be directly associated with the severity of cervical lesions [19, 26]. Dona *et al.* reported the association between p16/Ki-67 positivity and HPV16 and/or 18 infection was two-fold stronger compared to that with the infection by other HR-HPV types (OR=9.92, 95% CI: 2.39–47.77 *vs*. OR=4.20, 95% CI: 0.99–20.87). p16/Ki-67 positivity resulted strongly associated with a CIN2+ diagnosis (OR=10.86 95% CI: 4.16–29.12) [26]. These data provide a basis for ASCUS triage, which is important for early detection and early intervention of cervical lesions.

Although the present study included a relatively small number of samples, in comparison with similar studies, it has several strengths. First, histopathology was the endpoint of the study, and nearly all of the women were evaluated using colposcopy and biopsy. Only 11 of women were excluded without colposcopy and biopsy. Previous studies have compared p16 single staining compared with HR-HPV for identifying underlying high-grade cervical dysplasia in cervical cytology [27, 28], however it is implemented p16/Ki67 dual-staining compared with HR-HPV in this study. Second, as a prospective study, HPV infection and p16/ki67 expression was taken before the colposcopy biopsy. Dual-staining cytology testing was performed from residual specimen out of the liquid-based cytology collected at the initial screening visit not follow-up, at short intervals, which reflexed the true situation of patients. Nevertheless, there were also some drawbacks to this study. The probability of HR-HPV infection under 30 years is high. Numerous studies have stratified the patients according age; however, the number of samples in this study was too small for stratification. In addition, as the collection of further follow-up data for the women with ASCUS is ongoing; no assessment of the longer-term predictive value of dual-staining cytology and HR-HPV testing can be made at this point in time.

In conclusion, p16/Ki67 dual-staining and HR-HPV can be used as an auxiliary monitoring index to triage ASCUS, which could improve the diagnosis of some patients referred for colposcopy and significantly decrease the colposcopy referral rate, reducing unnecessary examination and treatment. The results of this study further indicate that p16/Ki67 dual staining is more accurate than HR-HPV for identifying underlying high-grade lesions in patients with ASCUS, which supports the usefulness of p16/Ki67 dual

staining in ASCUS triage. However, a larger sample of clinical observations is needed to confirm this conclusion.

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