

Performance of HPV Genotyping Combined with p16/Ki-67 in Detection of Cervical Precancer and Cancer Among HPV-Positive Chinese Women

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ABSTRACT

Women with positive high-risk human papillomavirus (hrHPV) need efficient triage testing to determine colposcopy referrals. Triage strategies of combining p16/Ki-67 with extended HPV genotyping were evaluated in this study. In total, 899 women attending cervical cancer screening program and 858 women referred to colposcopy from five hospitals were recruited. All the participants were tested by HPV assays and p16/Ki-67 dual staining. Colposcopy and biopsy were performed on women with any abnormal results. HPV genotypes were divided into four strata (HPV16/18, HPV31/33/58/52, HPV45/59/56/66, and HPV51/39/68/35) according to their risks for cervical intraepithelial neoplasia grade 3 or worse (CIN3+). The positive rates of four genotype strata among CIN3+ women were 3.47% (HPV51/39/68/35), 7.73% (HPV45/59/56/66), 14.7% (HPV31/33/58/52), and 78.1% (HPV16/18), respectively ($P_{\text{trend}} < 0.001$). The positive rates of p16/Ki-67 increased

with the elevation of HPV risk hierarchical from 65.0% in HPV51/39/68/35-positive women to 88.0% in HPV16/18-positive women ($P_{\text{trend}} < 0.001$). p16/Ki-67 was an effective method for risk stratification of CIN2+ among HPV31/33/58/52- and HPV45/59/56/66-positive women [HPV31/33/58/52: OR for dual stain+ (OR_{DS+}) of 26.7 (16.8–42.4) and OR for dual stain– (OR_{DS-}) of 3.87 (1.89–7.91); HPV45/59/56/66: OR_{DS+} of 10.3 (5.05–21.0) and OR_{DS-} of 1.27 (0.38–4.26)]. The combination of HPV16/18 genotyping and p16/Ki-67 triage of HPV31/33/58/52/45/59/56/66-positive women resulted in a lower referral rate (40.1% vs. 41.3%; $P < 0.001$) as compared with triage of 12 other HPV-positive women with p16/Ki-67, although sensitivity and specificity levels for these two strategies were identical. Combining HPV extended genotyping and p16/Ki-67 can be considered as a promising strategy for cervical cancer screening and triage.

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Introduction

Human papillomavirus (HPV) DNA detecting of cervical precancer and cancer is confirmed an efficient method for cervical primary screening (1). The U.S. Preventive Services Task Force Recommendation Statement as well as Chinese Society for Colposcopy and Cervical Pathology of China Healthy Birth Science Association recommend screening with primary hrHPV testing alone in women ages 30–65 (2). Study conducted among the U.S. women found that primary hrHPV screening starting at 25 years of age doubled the number of colposcopies compared with the same strategy starting at 30 years of age (3). Although HPV testing is regarded as a primary screening tool in several countries (4, 5), it harbors potential harms such as excess colposcopy and overtreatment of nonneoplastic lesions (6). Most HPV infections were transient and asymptomatic that can be eliminated spontaneously after a few months (7). Therefore, appropriate triage strategies are needed to more accurately identify cervical cancer and precancer and stratify risk in HPV-positive women.

American Society for Colposcopy and Cervical Pathology screening guidelines have proposed a strategy for primary screening that refers all HPV16/18 genotyping-positive women to colposcopy and triage of women positive for 12 other

HPV genotypes using cytology (6, 8, 9). Cytology as a triage tool for hrHPV-positive women was limited by its poor reproducibility and the level of the cytologists (10, 11). p16/Ki-67 dual stain, independent of morphologic criteria, displayed good reproducibility (12) and could achieve better performance compared with cytology for triage of hrHPV-positive women (13, 14). When combined with HPV16/18 partial genotyping, p16/Ki-67 showed the highest sensitivity for cervical intraepithelial neoplasia grade 3 or worse (CIN3+) of all the triage strategies that were evaluated in the ATHENA trial (15). The 12 other HPV genotypes were not all in the same risk strata, which prompted our hypothesis that HPV genotyping could provide more information on management of hrHPV-positive women. BD Onclarity HPV assay, approved by the FDA for HPV16, 18, and 45 individually reporting and approved HPV extended genotyping in Europe, has nine typing channels: HPV16, HPV18, HPV31, HPV45, HPV51, HPV52, HPV33/58, HPV35/39/68, and HPV56/59/66 (16). Previous research showed that HPV testing with Onclarity could be combined with cytology to predict risks of cervical precancer and cancer (17). It remains unclear whether the combination of p16/Ki-67 and extended HPV typing can serve as a powerful triage tool for HPV-positive women.

To evaluate the triage strategy of combining p16/Ki-67 and extended HPV genotyping for detecting CIN2/3+, we launched this case-enriched, multicenter study in China.

Materials and Methods

Study population and procedure

Study flow diagram is shown in Fig. 1. This is a cross-sectional, multicenter study. From April 2014 to March 2015, 971 women participating in cervical cancer screening program and 995 women attending colposcopy clinics were recruited [the five hospitals included: National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (NCC); Shanxi Cancer Hospital (SCH); Tianjin Central Hospital of Gynecology and Obstetrics (TCH); West China Second University Hospital, Sichuan University (SAH); Henan Cancer Hospital (HCH)]. Women from screening program signed the informed consent and met the following criteria: older than 25 years, had a cervix, had not been previously diagnosed with cervical cancer/precancer, and not pregnant. Women who previously received treatment for cervical diseases (such as hysterectomy or destructive therapy) were excluded. Women from colposcopy clinics based on abnormal cytology results or abnormal cytology results in combination with hrHPV positive or other clinical suspicion of cervical cancer and were clinically biopsy-confirmed CIN2+ locally, besides, they also signed the informed consent and met the inclusion/exclusion criteria above.

The cervical samples of all women were obtained and then kept in PreservCyt Solution (Hologic Inc.), stored at 4°C until transported to central laboratory of NCC for HPV DNA test (cobas4800 HPV test), liquid-based cytology (LBC) detection, OncoE6 test, and p16/Ki-67 dual staining detection. Women

from screening site with positive results for any test [positive for cobas or p16/Ki-67 or OncoE6 or cytologic \geq atypical squamous cells of undetermined significance (ASC-US)] were called back for colposcopy and biopsy. Women from colposcopy clinics were recruited for cervical LBC samples collection before treatment. Residue samples were stored at ultralow temperature refrigerator (-78°C) in central laboratory of NCC until tested for extended HPV genotyping by BD Onclarity HPV assay in 2016. Blinded testing with BD Onclarity was performed by technicians in NCC. The study was approved by Institutional Review Board of NCC following ethical guidelines of Declaration of Helsinki (approval number of NCC is 13-088/764).

In this analysis, 127 women were excluded because of unsatisfactory pathologic diagnosis ($n = 42$) and invalid tests ($n = 90$, 30 samples for p16/Ki-67 dual stain and 60 samples for BD Onclarity HPV assay). Women younger than 30 years were excluded ($n = 82$). A total of 1,757 women were included in the analysis (899 women were from screening population and 858 were clinical patients).

HPV DNA testing

The residual specimens were tested by BD Onclarity HPV assay performing on fully automated Viper LT system according to the manufacturer's instructions. BD Onclarity, a real-time PCR assay targeting on the HPV DNA E6/E7 regions, requires 0.5 mL specimens input and reports nine typing channels, including HPV16, HPV18, HPV45, HPV52, HPV51, HPV31, HPV33/58, HPV35/68/39, and HPV59/56/66.

LBC and p16/Ki-67 dual staining cytology

Cytology was performed using the ThinPrep System (Hologic Inc.). Two slides were prepared from one specimen according to the manufacturer's instructions. For LBC, slides were stained by Papanicolaou method, and cytologically classified according to the Bethesda System (2001). Cytologic atypical squamous cells of undetermined significance (ASC-US) or worse was defined as LBC abnormal.

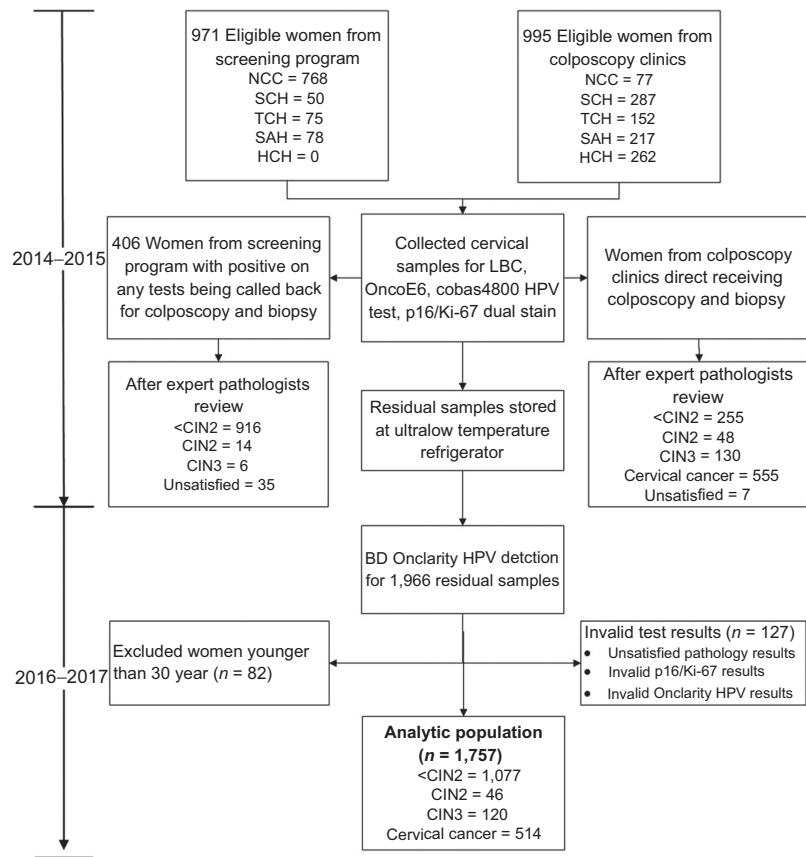
For p16/Ki-67 dual stain, slides were stained by CINtec PLUS Cytology Kit (Roche Tissue Diagnostics/Ventana Medical Systems, Inc.) according to the manufacturer's instructions. The presence of one or more cells showing simultaneous nuclear red Ki-67 staining and brown cytoplasmic p16 staining was considered as positive p16/Ki-67 dual stain. All the slides were reviewed by trained cytotechnologists at NCC, blinded to other test results.

Histopathologic diagnoses

Histopathologic slides were interpreted by local pathologists first, and then all CIN as well as HPV-negative cervical cancer cases, including squamous cell carcinoma and adenocarcinoma, were selected to be stained using p16INK4A IHC (Roche Tissue Diagnostics/Ventana Medical Systems, Inc.). In addition, all the adenocarcinoma cases were selected for progesterone receptor IHC (ZSGB-BIO). All the selected slides above, including histologic and IHC slides, were reviewed by a panel of

Figure 1.

Flow diagram of the study population. There are 406 women from screening program who were called back for colposcopy and biopsy, the rest of women ($n = 565$) were deemed to be without CIN lesions.



expert pathologists who gave the first interpretation from each center. The final diagnosis was determined by the panel of expert pathologists based on group discussion.

Statistical analysis

For the first analysis, HPV hierarchical analysis was applied to group HPV genotypes into four strata according to CIN3+ risk [group A: OR > 10; group B: OR > 1 (95% confidence interval (CI) did not include 1); group C: OR ≈ 1 (95% CI included 1); group D: OR ≤ 1 (point estimation lower than 1)]. At each step of the hierarchical analysis, we excluded the groups with the higher risk as previous studies described (18). Univariate logistic model was employed for estimating OR with 95% CI. χ^2 Trend test was used to evaluate the p16/Ki-67 dual stain positivity in histology categories and HPV type strata. For Fig. 2, OR represented the odds of positive genotype among CIN3+ cases dividing odds of negative test for same genotype among CIN3+ cases. For Fig. 3, OR represented the odds of positive genotype among CIN2+ (A) or CIN3+ (B) cases dividing odds of HPV negative among CIN2+ (A) or CIN3+ (B) cases. The clinical performance of different triage strategies [A, p16/Ki-67 alone for triage of 14 pooled genotypes HPV-positive women; B, combined HPV16/18 genotyping with p16/Ki-67 triage of women positive for 12 other HPV genotypes and negative for HPV16/18; and C, combined HPV16/18 genotyping with p16/Ki-67 triage of women positive for eight other

HPV genotypes (HPV31/33/58/52/45/59/56/66) and negative for HPV16/18]] were evaluated by sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and referral rates. McNemar test was used to evaluate the

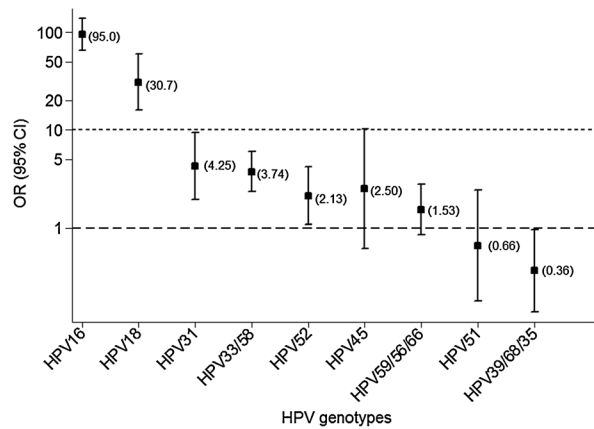


Figure 2.

Association of HPV genotypes and CIN3+. Boxes with vertical lines indicated point estimates of OR with 95% CI. The dotted line indicates OR of 10, and the dashed line indicated OR of 1. The total analysis sample size was 1,757 ($n_{\text{HPV16-positive}} = 497, n_{\text{HPV18-positive}} = 66, n_{\text{HPV31-positive}} = 40, n_{\text{HPV33/58-positive}} = 132, n_{\text{HPV52-positive}} = 64, n_{\text{HPV45-positive}} = 15, n_{\text{HPV59/56/66-positive}} = 115, n_{\text{HPV51-positive}} = 26, \text{ and } n_{\text{HPV39/68/35-positive}} = 58$).

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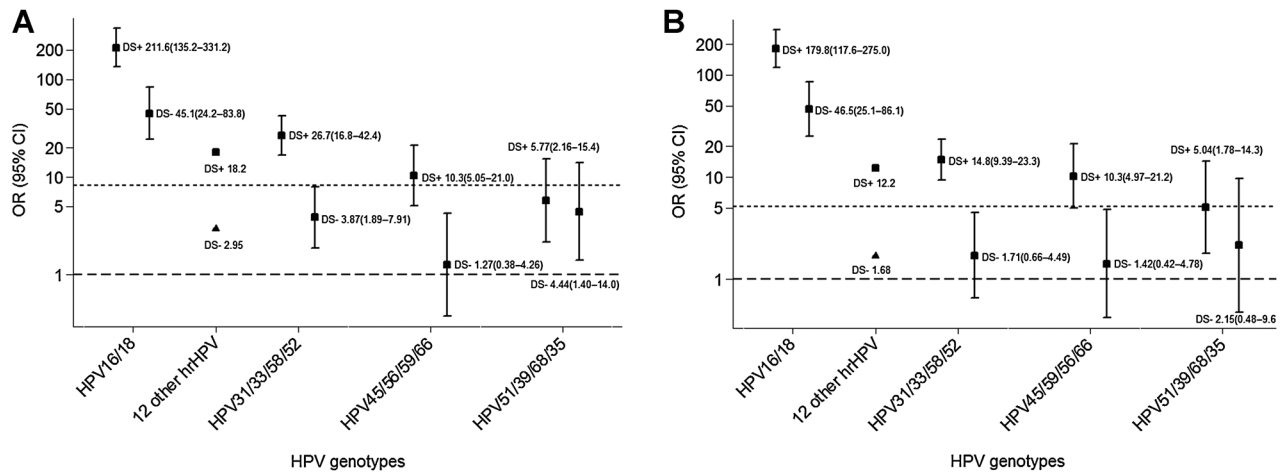


Figure 3. Risk of cervical cancer and precancer according to p16/Ki-67 results among HPV genotype-positive women. Risk of CIN2+ (A) and CIN3+ (B) for p16/Ki-67 positive in relation to p16/Ki-67 negative among HPV-positive women. Boxes with vertical lines indicate point estimates of OR with 95% CI. The square indicates point estimate of OR for p16/Ki-67-positive women among 12 other hrHPV-positive women. The triangle indicates point estimate of OR for p16/Ki-67-negative women among 12 other hrHPV-positive women. The dotted line indicates 8.28 (A) or 5.18 (B), which were OR of HPV-positive and cytologic ASC-US women who should undergo immediate colposcopy. The dashed line indicates OR of 1, higher than which means a risk factors.

difference of referral rates between different triage strategies. Two-side tests were chosen with α level adjusted to 0.0167 according to Bonferroni adjustment. All statistical analyses were run in Stata 14.0 (StataCorp).

Results

Study population

Table 1 shows the sociodemographics of 1,757 women. The mean age of entire population was 48.8 years. Most of women

Table 1. Sociodemographics by study population.

	Population category		Total (N = 1,757)
	Screening population (n = 899)	Clinics referral (n = 858)	
Age (mean ± SD)	50.2 ± 8.23	47.3 ± 9.26	48.8 ± 8.86
Menopause			
Yes	361 (40.2)	361 (40.2)	673 (38.3)
No	538 (59.8)	546 (63.6)	1,084 (61.7)
Gravidity			
0	9 (1.00)	44 (5.13)	53 (3.02)
1 or 2	282 (31.4)	262 (30.5)	544 (31.0)
≥3	608 (67.6)	552 (64.3)	1,160 (66.0)
Parity			
0	10 (1.11)	53 (6.18)	63 (3.59)
1 or 2	605 (67.3)	525 (61.2)	1,130 (64.3)
≥3	284 (31.6)	280 (32.6)	564 (32.1)
Smoking			
Yes	9 (1.00)	55 (6.4)	64 (3.6)
No	890 (99.0)	803 (93.6)	1,693 (96.4)
Drinking ^a			
Yes	79 (8.80)	166 (19.9)	245 (14.1)
No	819 (91.2)	670 (80.2)	1,489 (85.9)

^aTwenty-three women missing data of drinking were excluded from the analysis.

were nonsmokers (96.4%) and nondrinkers (85.9%). Among 1,757 women, 1,160 (66.0%) were gravidities 3 times or more, and 1,130 (64.3%) were parities 1 or 2 times.

Hierarchical risk of HPV genotypes

HPV genotypes were divided into four risk strata according to the associations between HPV genotypes and odds of CIN3+ for the dataset that was analyzed (Fig. 2). HPV16 and HPV18 were in group A, with OR of 95.0 (95% CI, 65.5–137.8) and 30.7 (95% CI, 15.9–59.5), respectively. Group B included HPV31 [4.25 (1.93–9.36)], HPV33/58 [3.74 (2.33–5.99)], and HPV52 [2.13 (1.08–4.18)]. HPV45 and HPV59/56/66 were in group C [HPV45 with OR of 2.50 (0.61–10.2) and HPV59/56/66 with OR of 1.53 (0.85–2.77)], and the rest HPV types were in the group D (HPV51 OR: 0.66 with 95% CI, 0.18–2.43 and HPV39/68/35 OR, 0.36 with 95% CI, 0.14–0.96).

The p16/Ki-67 positivity by histology and HPV risk stratification

Of the 1,757 participants in the study, 832 (47.4%) were hrHPV positive (Table 2). The percent of HPV-positive women in screening population was 18.1% and 78.0% in clinics patients. Among the entire population, the positive rate of HPV16/18 increased from pathologic non-neoplasia (3.86%) and CIN1 (12.1%), through CIN2 (21.7%) and CIN3 (65.8%), to cervical cancer (80.9%), while the rest of HPV types did not present the same tendency ($P < 0.001$). The positive rates of four HPV genotype strata among CIN3+ women were 3.47% (HPV51/39/68/35), 7.73% (HPV45/59/56/66), 14.7% (HPV31/33/58/52), and 78.1% (HPV16/18), respectively ($P_{\text{trend}} < 0.001$). The upward trend of p16/Ki-67 positivity with the severity of histopathology was observed, and the corresponding positive rate was

Table 2. HPV and p16/Ki-67 distribution by using histology stratification.

HPV genotypes	p16/Ki-67	Normal ^a	CIN1	CIN2	CIN3	Cervical cancer ^b	CIN2+	CIN3+	Total
		(n = 1,011) N (%)	(n = 66) N (%)	(n = 46) N (%)	(n = 120) N (%)	(n = 514) N (%)	(n = 680) N (%)	(n = 634) N (%)	(N = 1,757) N (%)
hrHPV ^{-c}	Total	835 (82.6)	30 (45.5)	6 (13.0)	10 (8.33)	44 (8.56)	60 (8.82)	54 (8.52)	925 (52.6)
	DS+	96 (11.5)	14 (46.7)	2 (33.3)	6 (60.0)	25 (56.8)	33 (55.0)	31 (57.4)	143 (15.5)
	DS-	739 (88.5)	16 (53.3)	4 (66.7)	4 (40.0)	19 (43.2)	27 (45.0)	23 (42.6)	782 (84.5)
hrHPV ^{+c}	Total	176 (17.4)	36 (54.6)	40 (87.0)	110 (91.7)	470 (91.4)	620 (91.2)	580 (91.5)	832 (47.4)
	DS+	88 (50.0)	20 (55.6)	31 (77.5)	99 (90.0)	422 (89.8)	552 (88.9)	521 (89.8)	660 (79.3)
	DS-	88 (50.0)	16 (44.4)	9 (22.5)	11 (10.0)	48 (10.2)	68 (11.1)	59 (10.2)	172 (20.7)
HPV16/18+	Total	39 (3.86)	8 (12.1)	10 (21.7)	79 (65.8)	416 (80.9)	505 (74.2)	495 (78.1)	552 (31.4)
	DS+	27 (69.2)	4 (50.0)	9 (90.0)	71 (89.9)	375 (90.1)	455 (90.1)	446 (90.1)	486 (88.0)
	DS-	12 (30.8)	4 (50.0)	1 (10.0)	8 (10.1)	41 (9.9)	50 (9.9)	49 (9.9)	66 (12.0)
HPV31/33/58/52+	Total	77 (7.62)	15 (22.7)	29 (63.0)	36 (30.0)	57 (11.1)	122 (17.9)	93 (14.7)	214 (12.2)
	DS+	38 (49.4)	8 (53.3)	23 (79.3)	34 (94.4)	53 (93.0)	110 (90.2)	87 (93.5)	156 (72.9)
	DS-	39 (50.7)	7 (46.7)	6 (20.7)	2 (5.56)	4 (7.00)	12 (9.80)	6 (6.50)	58 (27.1)
HPV45/59/56/66+	Total	59 (5.84)	12 (18.2)	6 (13.0)	11 (9.17)	38 (7.39)	55 (8.09)	49 (7.73)	126 (7.17)
	DS+	28 (47.5)	6 (50.0)	5 (83.3)	10 (90.9)	32 (84.2)	47 (85.5)	42 (85.7)	81 (64.3)
	DS-	31 (52.5)	6 (50.0)	1 (16.7)	1 (9.10)	6 (15.8)	8 (14.5)	7 (14.3)	45 (35.7)
HPV51/39/68/35+	Total	40 (3.96)	11 (16.7)	7 (15.2)	12 (10.0)	10 (1.95)	29 (4.26)	22 (3.47)	80 (4.55)
	DS+	20 (50.0)	8 (72.7)	5 (71.4)	10 (83.3)	9 (90.0)	24 (82.8)	19 (86.4)	52 (65.0)
	DS-	20 (50.0)	3 (27.3)	2 (28.6)	2 (16.7)	1 (10.0)	6 (20.7)	3 (13.6)	28 (35.0)
Total	Total	1,011 (57.5)	66 (3.76)	46 (2.62)	120 (6.83)	514 (29.3)	680 (38.7)	634 (36.1)	1,757 (100)
	DS+	184 (18.2)	34 (51.5)	33 (71.7)	105 (87.5)	447 (87.0)	585 (86.0)	552 (87.1)	803 (45.7)
	DS-	827 (81.8)	32 (48.5)	13 (28.3)	15 (12.5)	67 (13.0)	95 (14.0)	82 (12.9)	954 (54.3)

Note: The cross-tab includes positivity of p16/Ki-67 and HPV genotypes by histology, and positivity of p16/Ki-67 by histology and HPV results. The positivity of every HPV genotype was computed without multiple infection exclusion. P_{trend} of p16/Ki-67+ across HPV risk stratification (excluding HPV+): $P < 0.001$; P_{trend} of p16/Ki-67 across histology categories: $P < 0.001$; P_{trend} of HPV genotypes across cervical cancer and CIN2/3+: $P < 0.001$; P_{trend} of HPV16/18 across histology categories: $P < 0.001$; P_{trend} of HPV31/33/58/52 across histology categories: $P < 0.001$; P_{trend} of HPV45/59/56/66 across histology categories: $P = 0.214$; and P_{trend} of HPV51/39/68/35 across histology categories: $P = 0.307$.

^aThe normal category includes women in the screening population who were not referral for colposcopy.

^bInclude squamous cell carcinoma and adenocarcinoma.

^cFourteen genotypes of hrHPV include HPV16, HPV18, HPV31, HPV33/58, HPV52, HPV45, HPV59/56/66, HPV51, and HPV39/68/35.

18.2%, 51.5%, 71.7%, 87.5%, and 87.0% for each histology category, respectively ($P_{trend} < 0.001$). The positive rates of p16/Ki-67 increased with the elevation of HPV risk hierarchical from 65.0% in HPV51/39/68/35-positive women to 88.0% in HPV16/18-positive women ($P_{trend} < 0.001$; Table 2).

Risk stratification of p16/Ki-67 for different HPV genotype-positive women

The risk stratification of p16/Ki-67 for different genotypes positive women are detailed in Fig. 3. Among HPV16/18-positive women, the OR of p16/Ki-67-positive patients for CIN2+ was significantly higher than that of p16/Ki-67-negative ones, both of which exceeded the OR (8.28) of ASC-US HPV-positive women for CIN2+ in current population (internal benchmark; Fig. 3A). ORs for p16/Ki-67 positive and negative were similar among women with HPV51/39/68/35 positive. For the rest of HPV genotypes (HPV31/33/58/52 and HPV45/59/56/66), p16/Ki-67 was capable of stratifying risk for CIN2+ [for HPV31/33/58/52 with OR for dual stain+ (OR_{DS+}) of 26.7 (16.8–42.4) and OR_{DS-} of 3.87 (1.89–7.91); for HPV45/59/56/66 with OR_{DS+} of 10.3 (5.05–21.0) and OR_{DS-} of 1.27 (0.38–4.26; Fig. 2A)]. Dual stain showed similar results for detection of CIN3+ (Fig. 2B).

Evaluation of triage strategies for hrHPV-positive women

Detailed results of triage strategies for triaging hrHPV-positive women are presented in Table 3 [A, p16/Ki-67 alone for triage of 14 pooled genotypes HPV-positive women; B, combined HPV16/18 genotyping with p16/Ki-67 triage of women positive for 12 other HPV genotypes (HPV31/33/58/52/45/59/56/66)]. Compared with performance of strategy A, higher sensitivity was obtained with strategy B and C in entire population and colposcopy clinics' patients for detection of both CIN2+ and CIN3+ [detection of CIN2+ in entire population: sensitivity (A): 89.0% (86.3–91.4%) vs. sensitivity (B): 97.1% (95.5–98.3%) vs. sensitivity (C): 96.1% (94.3–97.5%), detection of CIN3+ in entire population: sensitivity (A): 89.8% (87.1–92.2%) vs. sensitivity (B): 98.3%(96.9–99.2%) vs. sensitivity (C): 97.4% (95.8–98.5%), detection of CIN2+ in clinical patients: sensitivity (A): 89.5% (86.8–91.8%) vs. sensitivity (B): 97.7% (96.1–98.7%) vs. sensitivity (C): 96.7% (94.9–98.0%), and detection of CIN3+ in clinical patients: sensitivity (A): 89.9% (87.1–92.2%) vs. sensitivity (B): 98.3% (96.8–99.2%) vs. sensitivity (C): 97.4% (95.7–98.5%)]. Furthermore, strategy C required lower referral rate ($P < 0.001$ for all) as compared with strategy B, although sensitivity and specificity levels for

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Table 3. Clinical performance of different triage strategies for detection of CIN2+ and CIN3+ among HPV-positive women.

Population	Strategy	TP	FP	TN	FN	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	N (%)	Referral rate ^a	
											P ^b	P ^b
Entire population N = 832												
CIN2+ n = 620												
A ^c	552	108	104	68	89.0 (86.3-91.4)	49.1 (42.1-56.0)	-	-	-	660 (37.6)	Reference	-
B ^d	602	124	88	18	97.1 (95.5-98.3)	41.5 (34.8-48.5)	-	-	-	726 (41.3)	<0.001	Reference
C ^e	596	109	103	24	96.1 (94.3-97.5)	48.6 (41.7-55.5)	-	-	-	705 (40.1)	<0.001	<0.001
CIN3+ n = 580												
A ^c	521	139	113	59	89.8 (87.1-92.2)	44.8 (38.6-51.2)	-	-	-	660 (37.6)	Reference	-
B ^d	570	156	96	10	98.3 (96.9-99.2)	38.1 (32.1-44.4)	-	-	-	726 (41.3)	<0.001	Reference
C ^e	565	140	112	15	97.4 (95.8-98.5)	44.4 (38.2-50.8)	-	-	-	705 (40.1)	<0.001	<0.001
Screening population N = 163												
CIN2+ n = 20												
A ^c	15	71	72	5	75.0 (50.9-91.3)	50.3 (41.9-58.8)	17.4 (10.1-27.1)	93.5 (85.5-97.9)	86 (9.57)	Reference	-	-
B ^d	16	80	63	4	80.0 (56.3-94.3)	44.1 (35.8-52.6)	16.7 (9.84-25.6)	94.0 (85.4-98.3)	96 (10.7)	0.002	Reference	-
C ^e	16	72	71	4	80.0 (56.3-94.3)	49.7 (41.2-58.1)	18.2 (10.8-27.8)	94.7 (86.9-98.5)	88 (9.79)	0.815	0.008	Reference
CIN3+ n = 6												
A ^c	5	81	76	1	83.3 (35.9-99.6)	48.4 (40.4-56.6)	5.81 (1.91-13.0)	98.7 (93.0-100)	86 (9.57)	Reference	-	-
B ^d	6	90	67	0	100 (54.1-100)	42.7 (34.8-50.8)	6.25 (2.33-13.1)	100 (94.6-100)	96 (10.7)	0.002	Reference	-
C ^e	6	82	75	0	100 (54.1-100)	47.8 (39.7-55.9)	6.82 (2.54-14.3)	100 (95.2-100)	88 (9.79)	0.815	0.008	Reference
Clinical patients N = 669												
CIN2+ n = 600												
A ^c	537	37	32	63	89.5 (86.8-91.8)	46.4 (34.3-58.8)	-	-	-	574 (66.7)	Reference	-
B ^d	586	44	25	14	97.7 (96.1-98.7)	36.2 (25.0-48.7)	-	-	-	630 (73.4)	<0.001	Reference
C ^e	580	37	32	20	96.7 (94.9-98.0)	46.4 (34.3-58.8)	-	-	-	617 (71.9)	<0.001	<0.001
CIN3+ n = 574												
A ^c	516	58	37	58	89.9 (87.1-92.2)	38.9 (29.1-49.5)	-	-	-	574 (66.7)	Reference	-
B ^d	564	66	29	10	98.3 (96.8-99.2)	30.5 (21.5-40.8)	-	-	-	630 (73.4)	<0.001	Reference
C ^e	559	58	37	15	97.4 (95.7-98.5)	38.9 (29.1-49.5)	-	-	-	617 (71.9)	<0.001	<0.001

Note: The difference of referral rates between two triage strategies were tested by McNemar χ^2 test or Exact McNemar test.

Abbreviations: FN, false negative; FP, false positive; TP, true positive; TN, true negative.

^aReferral rates were calculated for entire population instead of limited in HPV-positive women.

^bAdjusted $\alpha = 0.0167$ (Bonferroni adjustment).

^cp16/Ki-67 alone for triage of HPV-positive women (14 pooled genotypes).

^dCombining HPV16/18 genotyping with p16/Ki-67 triage of women positive for 12 other HPV genotypes.

^eCombining HPV16/18 genotyping with p16/Ki-67 triage of women positive for eight other HPV genotypes (HPV31/33/58/52/45/59/56/66).

these two strategies were identical among entire population and colposcopy clinics' patients.

Discussion

Efficient triage strategies are needed if primary HPV screening is used at the national level. Currently, there is no obvious winning triage strategy for primary HPV screening (19, 20), even though triage methods of OncoE6, methylation, viral load, p16/Ki-67, and HPV genotyping have been studied for years. Following our literature survey, this is the first study to explore performance of combining p16/Ki-67 with extended HPV genotyping for detecting CIN2/3+ among Chinese women. Our results indicated that the combination of HPV16/18 genotyping and p16/Ki-67 triage of women positive for eight other HPV genotypes was a promising approach. Furthermore, the strategy presented lower referral rate and similar sensitivity and specificity as compared with combining HPV16/18 genotyping and p16/Ki-67 triage of women positive for 12 other HPV genotypes.

In our study, HPV genotypes were divided into four strata (HPV16/18, HPV31/33/58/52, HPV45/59/56/66, and HPV51/39/68/35) according to their risks for CIN3+. HPV16/18 dominated in cervical cancer, while other hrHPV types accounted for a large proportion in CIN1/2 and their contribution to cervical cancer dropped steeply, especially HPV51/39/68/35. A meta-analysis showed that HPV51/39/68/35 had low invasive cervical cancer: normal ratios, suggesting the low carcinogenic potential of these types (21).

Data from Kaiser Permanente Northern California (KPNC) study found that combined extended HPV genotyping in primary screening and reflex cytology to stratify risk of CIN3+ is quite powerful (17). We found p16/Ki-67 was capable of risk stratification for HPV16/18 and eight other HPV genotypes (HPV31/33/58/52/45/59/56/66), but not for HPV51/39/68/35 within a Chinese population. The p16/Ki-67 was an effective triage tool only for women positive for eight other HPV genotypes, because HPV16/18-positive women displayed sufficiently high risk of CIN3+ for immediate colposcopy, even with p16/Ki-67 negative. Our previous study showed that p16 could be implemented as a potent biomarker for prediction of cervical lesion progression (22) and that p16/Ki-67 associated strongly with hrHPV persistence (23), which is linked to an increased risk of subsequent cancer development. In this study, risk of p16/Ki-67 positive was similar to that of p16/Ki-67 negative among HPV51/39/35/68-positive women. It seems probable that women with HPV51/39/35/68 positive have lower probability of cervical lesion progression (24), even if these women have CIN2. Only 20% of CIN2 would progress to CIN3+ in 5 years (25) and the 30-year progression rate of CIN3 to cancer was about 31% (26).

The ATHENA trial candidate algorithm modeling demonstrated HPV16/18-positive women should undergo immediate colposcopy (27), and the combination of HPV16/18 genotyping

and cytology became a standard triage strategy for primary hrHPV screening (6). Canadian Cervical Cancer Screening Trial reported that referring all HPV16/18-positive and HPV16/18-negative, but ASC-US+ women had the highest sensitivity among all studied strategies, as expectedly, superior sensitivity coming at a cost in terms of specificity (28). p16/Ki-67 is superior to cytology, with higher sensitivity (ATHENA) and specificity (KPNC and PROTECT-3B), for triage of hrHPV-positive women (13–15, 29, 30). Our data confirmed the high risk of precancer and cancer in HPV16/18-positive women, suggesting the necessity of immediate colposcopy for them. Managing women with HPV16/18 negative but 12 other HPV positive is more challenging and still important, and subsequent negative p16/Ki-67 among these women led to a posttest CIN2+ risk levels of <2% (31), which is below the threshold for retesting. We found that triage strategy of combining HPV16/18 genotyping with p16/Ki-67 provided higher sensitivity than that of triage by p16/Ki-67 alone among HPV-positive women, which was similar with Wright and colleagues' study, but they also observed lower specificity (15). Moreover, referral of all HPV16/18-positive women combined with p16/Ki-67 triage of women positive for eight other HPV genotypes provided a similar clinical performance as triage of 12 other HPV genotypes, while the former had relatively lower referral rate. Therefore, the combination of HPV16/18 genotyping and p16/Ki-67 triage of eight other HPV genotypes is an effective strategy, which seems likely to be a promising way for cervical cancer screening in low- and middle-income countries (LMIC). Although visual inspection with acetic acid (VIA) has been widely used in LMICs, it is inferior to cytology as a triage tool among HPV-positive women (32). However, it is not practical to propose a screening program with cytology in LMICs, because there are still problems needing resolution: (i) shortage of trained cytologists, especially in developing areas; (ii) the poor reproducibility of cytologic diagnosis; and (iii) being more costly for screening by cytology than VIA and HPV testing (33). The p16/Ki-67 bypasses the need for morphologic evaluation, suggesting that it can be implemented with limited training and that it may have high reproducibility among cytotechnologists. Moreover, the new triage strategy proposed by our study could reduce the number of dual stain assessments. However, the costs of the dual stain and extended HPV genotyping products used in our study are currently estimated to be too high for LMICs. This suggests that suitable products of dual stain and HPV genotyping could be developed for LMICs. Further analysis based on cost-effectiveness need to be conducted to confirm the prospect of the new strategy in LMICs.

Our study has some important strengths. First, the disease ascertainment was excellent because the outcomes in the screening population were obtained through biopsy of all women positive for any of four tests. Furthermore, we enriched amounts of cervical cancer might clarify the

contribution of HPV and p16/Ki-67 more than precancer (CIN2/3), which contain probabilities of lesions regression. However, this case-enriched study was a double-edged sword and it also brought some limitations to our research. We studied the triage strategy on a combined population to enrich an amount of CIN2+ cases so the risks for CIN2/3+ were overestimated among the entire population. The specific data from entire population cannot directly be translated to real clinical situation and the extrapolation of the study was restricted. However, the comparison among triage strategies within the study population was meaningful and reliable. Previous data from ATHENA and PROTECT-3B have confirmed the combination of HPV16/18 genotyping and reflex p16/Ki-67 triage of women positive for 12 other HPV genotypes was a reliable method for referring primary HPV-positive women. The results of our study were in agreement with Wright and colleagues' study (15), suggesting reliability of current results for triage of HPV-positive women. Meanwhile, we provided data of clinical performance of the three strategies being separated by population sources. We found that data from entire population were similar with that of colposcopy clinics when clinical performances of strategies were evaluated.

In conclusion, we observed a good risk stratification of p16/Ki-67 in HPV31/33/58/52/45/59/56/66-positive women. There may be different management recommendations for women, based on genotype strata and dual stain results, according to the principle of "equal management of equal risk" (34). Moreover, the method of extended HPV genotyping combined with p16/Ki-67 for detecting CIN2/3+ could provide reliable clinical performance. Wentzensen and colleagues found that the screening interval for women with both HPV16/18 and p16/Ki-67 negative could be extended to 3 years (30). However,

subsequent large sample size cohort studies are needed to explore the retest interval in women with other HPV genotypes and p16/Ki-67 negative.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the other authors.

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