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Triage performance of *PAX1*^{m}/*JAM3*^{m} in opportunistic cervical cancer screening of non–16/18 human papillomavirus-positive women: a multicenter prospective study in China

Xiaojing Chen[1,2,3], Xitong Jin[4], Linghua Kong[1,2,3], Yuligh Liou[4,5], Pei Liu[4], Zhe Dong[6], Sijun Zhou[7], Bingli Qi[8], Jing Fei[9], Xiaoyan Chen[10], Guangwu Xiong[6], Yuchong Hu[7], Shikai Liu[8], Jianwei Zhou[9], Huafeng Shou[10] and Lei Li[1,2,3*]

Abstract

Objectives In this study, we aimed to validate the performance of the *PAX1* and *JAM3* methylation (*PAX1*^{m}/*JAM3*^{m}) test as a triage tool for detecting cervical intraepithelial neoplasia grade 3 or worse (CIN3 +) in non-16/18 high-risk human papillomavirus-positive patients (non-16/18 hrHPV +).

Methods The triage performance of liquid-based cytology (LBC) and the *PAX1*^{m}/*JAM3*^{m} test for detecting CIN3 + were compared.

Results In total, 1851 participants had cervical histological outcomes and were included in the analysis. The sensitivity/specificity of the LBC test results with atypical squamous cells of undetermined significance or worse (LBC ≥ ASCUS) and the *PAX1*^{m}/*JAM3*^{m} test were 90.1%/26.7% and 84.8%/88.5%, respectively. *PAX1*^{m}/*JAM3*^{m}(+) had the highest diagnostic AUC (0.866, 95% confidence interval (CI) 0.837–0.896) in the whole cohort. All cancers (n = 20) were detected by *PAX1*^{m}/*JAM3*^{m}(+). Compared with LBC ≥ ASCUS, *PAX1*^{m}/*JAM3*^{m}(+) reduced the number of patients who needed referral for colposcopy by 57.21% (74.66% vs. 17.45%). The odds ratios for detecting CIN3 + by LBC ≥ ASCUS and *PAX1*^{m}/*JAM3*^{m}(+) were 3.3 (95% CI 2.0–5.9) and 42.6 (27.1–69.6), respectively ($p < 0.001$). The combination of LBC ≥ ASCUS or *PAX1*^{m}/*JAM3*^{m}(+) slightly increased the diagnostic sensitivity (98.0%, 95% CI: 95.8–100%) and referral rate (77.09%) but reduced the diagnostic specificity (24.8%, 22.7–26.8%).

Conclusions In non-16/18 hrHPV(+) women, *PAX1*^{m}/*JAM3*^{m} was superior to cytology for detecting CIN3 +. Compared with LBC ≥ ASCUS, *PAX1*^{m}/*JAM3*^{m}(+) reduced the number of significant referrals to colposcopy without compromising diagnostic sensitivity.

Keywords Cervical cancer, Cervical intraepithelial lesions, Human papillomavirus DNA tests, Liquid-based cytology, DNA methylation, Non-16/18-type high-risk human papillomavirus infections

*Correspondence:
Lei Li
lileigh@163.com
Full list of author information is available at the end of the article

Introduction

Cervical cancer (CC) is a major public health issue affecting women globally and ranks fourth among cancers causing fatalities in females [1]. With advancements in vaccination, screening, and treatment [2], the risk of cervical cancer has been significantly reduced in recent years by early detection and treatment of precancerous cervical lesions [3, 4]. Additionally, cervical cancer screening and the triage of individuals with abnormal screening results are the most important aspects in the secondary prevention of cervical cancer [5, 6].

Current HPV screening strategies are limited by the overdetection of clinically irrelevant, transient HPV infections, which can result in unnecessary follow-up visits, colposcopy overreferral, and overtreatment [7]. The World Health Organization (WHO) recommends initial screening with HPV DNA testing in combination with specific triage methods and regular screening every 5 to 10 years [8, 9]. The lower specificity of HPV testing is currently countered by reflex triage testing with (repeat) cytology, especially in non-16/18 hrHPV-positive patients [7, 10]. Currently, the guidelines of the American Society for Colposcopy and Cervical Pathology (ASCCP) and the Chinese Society for Colposcopy and Cervical Pathology (CSCCP) directly refer to HPV16/18-positive women for colposcopy [11]. For non-16/18-type HPV infections, the guidelines recommend incorporating results from liquid-based cytology (LBC) testing for further consideration. Colposcopy and biopsy are recommended for LBC-positive (atypical squamous cells of undetermined significance (ASCUS) or worse (≥ASCUS) individuals, whereas follow-up is advised for LBC-negative patients [12]. However, LBC results are highly susceptible to the influence of subjective factors, significant variability between different pathologists and laboratories, and stringent training requirements [13, 14]. It is also affected by sampling, preparation, patient age, and the lesion itself [15]. Consequently, cervical cancer caused by non-16/18 hrHPV infections is the focus of future screening and treatment efforts.

Many studies have provided increasing evidence that DNA methylation testing can be a promising cervical screening method because of its good performance, reproducibility, and ability to triage hrHPV-positive (hrHPV+) women [16–23]. The *PAX1* gene, which contains a paired box domain and a paired-type homeodomain, is a member of the paired box (PAX) family. The *PAX1* gene plays a crucial role in spinal cord development and embryogenesis [24]. A higher *PAX1* methylation level promotes the deterioration of lesions and induces tumorigenesis, such as in cervical, oral, and esophageal cancer [25, 26]. *JAM* family members belong to the immunoglobulin superfamily and play important roles in maintaining tight junction integrity, regulating cell migration, and determining cellular polarity [27, 28]. The methylation status of the host genes *PAX1* and JAM3 has great potential for the triage of hrHPV-infected individuals in cervical cancer screening [16, 22, 23, 29, 30]. Previous studies have shown that, as a secondary triage modality for hrHPV(+) women, the sensitivity and specificity of *PAX1* methylation status alone or *PAX1* and *JAM3* in combination with detection of other genes for cervical intraepithelial neoplasia grade 3 or worse (CIN3+) can reach more than 80% [31, 32]. In addition, the triage effectiveness of DNA methylation testing is comparable to that of cytology, and even methylation triage can reduce the number of referred patients [10, 16]. For hrHPV(+) triage, the *PAX1/JAM3* methylation ($PAX1^m/JAM3^m$) detection kit (CISCER®) has been approved by the China National Medical Products Administration. However, its non-16/18 hrHPV(+) triage effectiveness has not been adequately explored.

To validate the triage effectiveness of $PAX1^m/JAM3^m$ in non-16/18 hrHPV(+), we enrolled participants from two prospective cohorts who received liquid-based cytology (LBC) testing, hrHPV testing, and $PAX1^m/JAM3^m$ testing for opportunistic cervical cancer screening. Non-16/18 hrHPV(+) participants were selected, and the triage efficacy of LBC with ≥ atypical squamous cells of undetermined significance (LBC ≥ ASCUS) and $PAX1^m/JAM3^m$ for detecting CIN3+ was assessed.

Materials and methods

Participants and study design

The participants were from two multicenter studies conducted from November 2020 to December 2021 (METHY3) and from May 2022 to October 2022 (METHY4). Eligible women underwent opportunistic cervical cancer screening consisting of hrHPV genotyping, LBC testing, and $PAX1^m/JAM3^m$ testing. The triage performance for detecting CIN3+ in non-16/18 hrHPV(+) women was compared according to the cervical histology results obtained via colposcopy or other surgical procedures.

The inclusion criteria for the two cohorts were the same and consisted of the following: 1) aged 18 years or older; 2) intact cervix; and 3) no serious immunodeficiency conditions, such as HIV infection, history of organ transplantation, or treatment with immunosuppressive drugs. Participants were excluded if they had known malignant tumors of the female genital tract or active malignant tumors of other sites still under treatment. All study subjects volunteered to participate in this study and signed an informed consent form.

Sample collection, cytologic pathology assay, and hrHPV genotyping

All eligible participants underwent gynecological examinations and collection of cervical cytology data. The cytology samples were subjected to hrHPV and cytological pathology assays. The residual samples were sent for $PAX1^m/JAM3^m$ testing.

All the samples were subjected to high-performance HPV testing via Cobas® 4800 assays (Roche Diagnostic Products, Shanghai, China). The Cobas HPV test was carried out according to the manufacturer's instructions [33]. The Cobas HPV test is an in vitro polymerase chain reaction-based method for quantitative detection of high-risk HPV DNA and can detect HPV16, HPV18, and non-16/18 hrHPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) of high-risk HPV types.

The LBC testing was used for cytology tests (Hologic, Inc., MA, USA). Cytologists used the 2014 Bethesda system [34] to classify LBC results. LBC diagnoses were classified as no intraepithelial lesions or malignancy (NILM), atypical squamous cells of undetermined significance (ASCUS); low-grade squamous intraepithelial lesion (LSIL); atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H); high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC) in this study.

DNA methylation assays

Methylation detection was performed in a certified DNA laboratory, and the operators and staff members were blinded to the clinical information, LBC testing, HPV genotyping, and cervical histopathology results of the patients. Genomic DNA (gDNA) was extracted from the exfoliated cervical sample with the JH-DNA Isolation and Purification Kit (OriginPoly Bio-Tec Co., Ltd., Beijing, China) per the manufacturer's instructions. The DNA concentration was quantified with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, DE, USA). Briefly, 200–1000 ng of gDNA was subjected to bisulfite conversion by means of JH-DNA Methylation-Lightning MagPrep (OriginPoly Bio-Tec Co., Ltd., Beijing, China). The levels of $PAX1^m$ and $JAM3^m$ were subsequently determined with the CISCER® DNA Methylation Detection Kit for Cervical Cancer (real-time PCR) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control (OriginPoly Bio-Tec Co., Ltd., Beijing, China) by means of the ABI 7500 real-time PCR system platform (Life Technology, Foster City, CA, USA) per the manufacturer's instructions. The hypermethylation level of the *PAX1* gene and *JAM3* gene was determined by the difference between the two Ct values (ΔCt *PAX1* = Ct *PAX1*-Ct *GAPDH* and ΔCt *JAM3* = Ct *JAM3*-Ct *GAPDH*). According to the manufacturer's instructions, a positive CISCER ($PAX1^m/JAM3^m$) test result was defined as a ΔCt $PAX1 \leq 6.6$ or ΔCt $JAM3 \leq 10.0$.

Histology evaluation

Cervical histology samples were collected by colposcopy performed according to current guidelines [35, 36] or by other surgical procedures, such as conization, the loop electrosurgical excision procedure (LEEP), or hysterectomy for cervical lesion indications. The results of $PAX1^m/JAM3^m$ were not considered indications for colposcopy referrals or cervical biopsy in this study.

Statistical analysis

SPSS 26.0 (IBM Corp., Armonk, NY, USA) and R (version 4.1.2, Vienna, Austria) were used for all the statistical analyses. The $\chi 2$ test was used for comparisons between groups. A normality test was used to determine whether the variance of the population was equal to that of the Kolmogorov–Smirnov test. Nonnormally distributed data are expressed as M (Q1, Q3), and comparisons between two groups were performed with the nonparametric Mann–Whitney U test. Receiver operating characteristic (ROC) curves were used to evaluate the AUCs of different detection methods between < CIN3 + patients and CIN3 + patients. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for detecting CIN3 + lesions were calculated with 95% confidence intervals (CIs). The sensitivity and specificity for detecting CIN3 + lesions between different triage methods were compared by means of using McNemar's test. All tests were two-sided, and P values < 0.05 with 95% CIs indicated statistically significant differences.

Results

Patient clinicopathological characteristics

As shown in Fig. 1, among a total of 30,084 women in six hospitals who underwent opportunistic cervical cancer screening, including methylation testing, 4735 participants (15.7%) tested non-16/18 hrHPV positive. The median age of all non-16/18 hrHPV(+) women was 42 years (interquartile range (IQR): 34–53). The cohort demographic characteristics and clinical information are summarized in Table 1. Details of each center are shown in the Additional file 1: Table.

In total, 1851 women had cervical histology results, and among them, 1121 (60.6%) had normal findings or inflammation, 401 (21.7%) had CIN1, 178 (9.6%) had CIN2, 131 (7.1%) had CIN3, and 20 (1.1%) had cervical cancer. All cancers (n = 20) were detected by $PAX1^m/JAM3^m$(+) testing. However, only 80% (16/20) of cancers with a diagnosis of ≥ ASCUS were

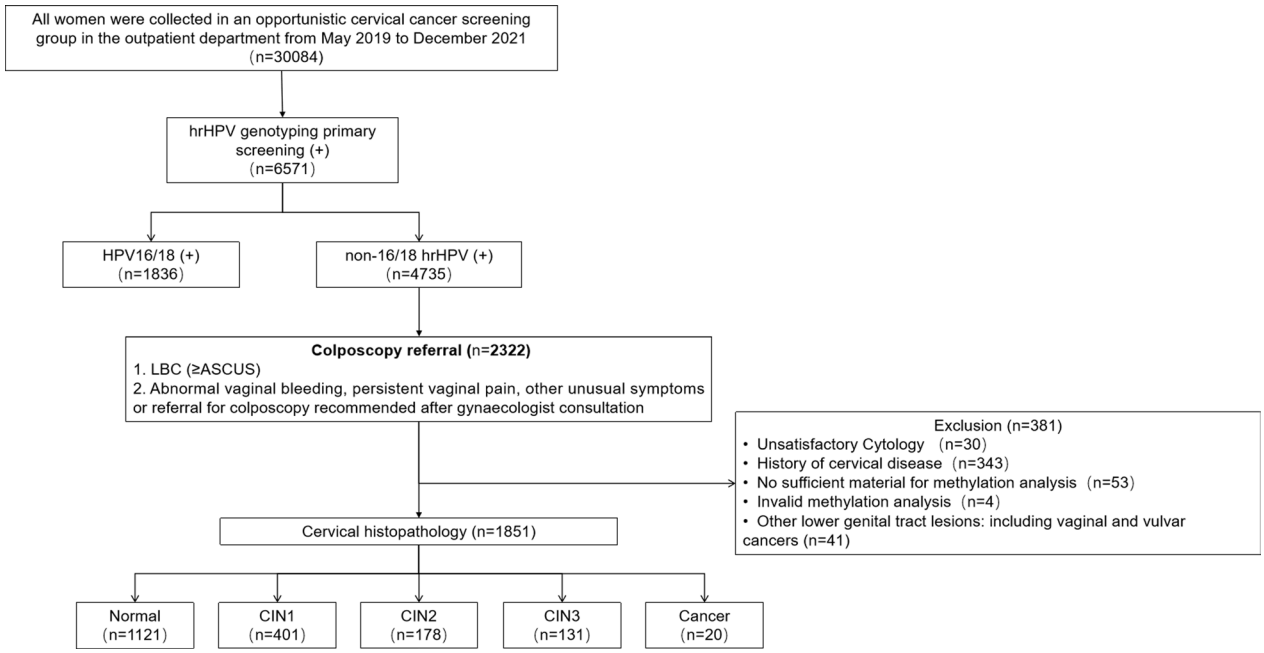

Fig. 1 Flowchart of the study. HPV 16/18(+), HPV16, and/or HPV18 positive; non-16/18 hrHPV(+), hrHPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 high-risk HPV types, infection with one or more of these HPV types; ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia

Table 1 Clinical Characteristics of different histopathology subgroups

Characteristics	Overall	Normal	CIN1	CIN2	CIN3	Cervical Cancer
n	1851	1121	401	178	131	20
AGE (median (IQR))	42.00 (34.00, 53.00)	43.00 (34.00, 55.00)	41.00 (33.00, 51.00)	39.50 (33.25, 48.00)	41.00 (35.00, 50.00)	51.00 (42.75, 61.25)
LBC Results (%)						
NILM	469 (25.3)	348 (31.0)	79 (19.7)	27 (15.2)	11 (8.4)	4 (20.0)
≥ ASCUS	1382 (74.7)	773 (69.0)	322 (80.3)	151 (84.8)	120 (91.6)	16 (80.0)
PAX1^m (%)						
(–)	1575 (85.1)	1084 (96.7)	375 (93.5)	79 (44.4)	36 (27.5)	1 (5.0)
(+)	276 (14.9)	37 (3.3)	26 (6.5)	99 (55.6)	95 (72.5)	19 (95.0)
JAM3^m (%)						
(–)	1601 (86.5)	1091 (97.3)	385 (96.0)	90 (50.6)	35 (26.7)	0 (0.0)
(+)	250 (13.5)	30 (2.7)	16 (4.0)	88 (49.4)	96 (73.3)	20 (100.0)
PAX1^m/*JAM3*^{m} (%)						
(–)	1528 (82.5)	1067 (95.2)	372 (92.8)	66 (37.1)	23 (17.6)	0 (0.0)
(+)	323 (17.5)	54 (4.8)	29 (7.2)	112 (62.9)	108 (82.4)	20 (100.0)
LBC ≥ ASCUS or P^m/J^m (+)						
(–)	424 (22.9)	332 (29.6)	75 (18.7)	14 (7.9)	3 (2.3)	0 (0.0)
(+)	1427 (77.1)	789 (70.4)	326 (81.3)	164 (92.1)	128 (97.7)	20 (100.0)
LBC ≥ ASCUS and P^m/J^m (+)						
(–)	1573 (85.0)	1083 (96.6)	376 (93.8)	79 (44.4)	31 (23.7)	4 (20.0)
(+)	278 (15.0)	38 (3.4)	25 (6.2)	99 (55.6)	100 (76.3)	16 (80.0)

CIN cervical intraepithelial neoplasia, *IQR* interquartile range, *LBC* liquid-based cytology test, *NILM* no intraepithelial lesions or malignancy; ≥ ASCUS: atypical squamous cells of undetermined significance or worse; *PAX1*^{m}(+): ΔCt *PAX1* ≤ 6.6; *JAM3*^{m}(+): ΔCt *JAM3* ≤ 10.0; *PAX1*^{m}/*JAM3*^{m}: ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS or P^m/J^m(+): LBC ≥ atypical squamous cells of undetermined significance or ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS and P^m/J^m(+): LBC ≥ atypical squamous cells of undetermined significance and ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM 3* ≤ 10.0

detected by LBC testing. Among the 4 cases of undetected cervical cancer by LBC, there were three cases of squamous cell carcinoma (SCC), and one case of adenocarcinoma (ADC). These four patients received referrals for colposcopy on the advice of their gynecologists because of abnormal vaginal bleeding. A total of 74.6% (1382/1851) and 17.5% (323/1851) of the whole cohort had LBC ≥ ASCUS and $PAX1^m/JAM3^m$ (+), respectively.

Relationships of *PAX1/JAM3* methylation with cervical histology and LBC results

The median ΔCt values of *PAX1* and *JAM3* methylation were significantly different among the pathological groups (Fig. 2A–B, $P < 0.05$). The median ΔCt of *PAX1* methylation significantly differed between NILM and ASCUS, between LSIL and AGC, and between ASC-H and HSIL (Fig. 2C–B, $P < 0.05$). The median ΔCt of *JAM3* methylation significantly differed between the ASC-H subgroup and the HSIL subgroup (Fig. 2D, $P < 0.05$).

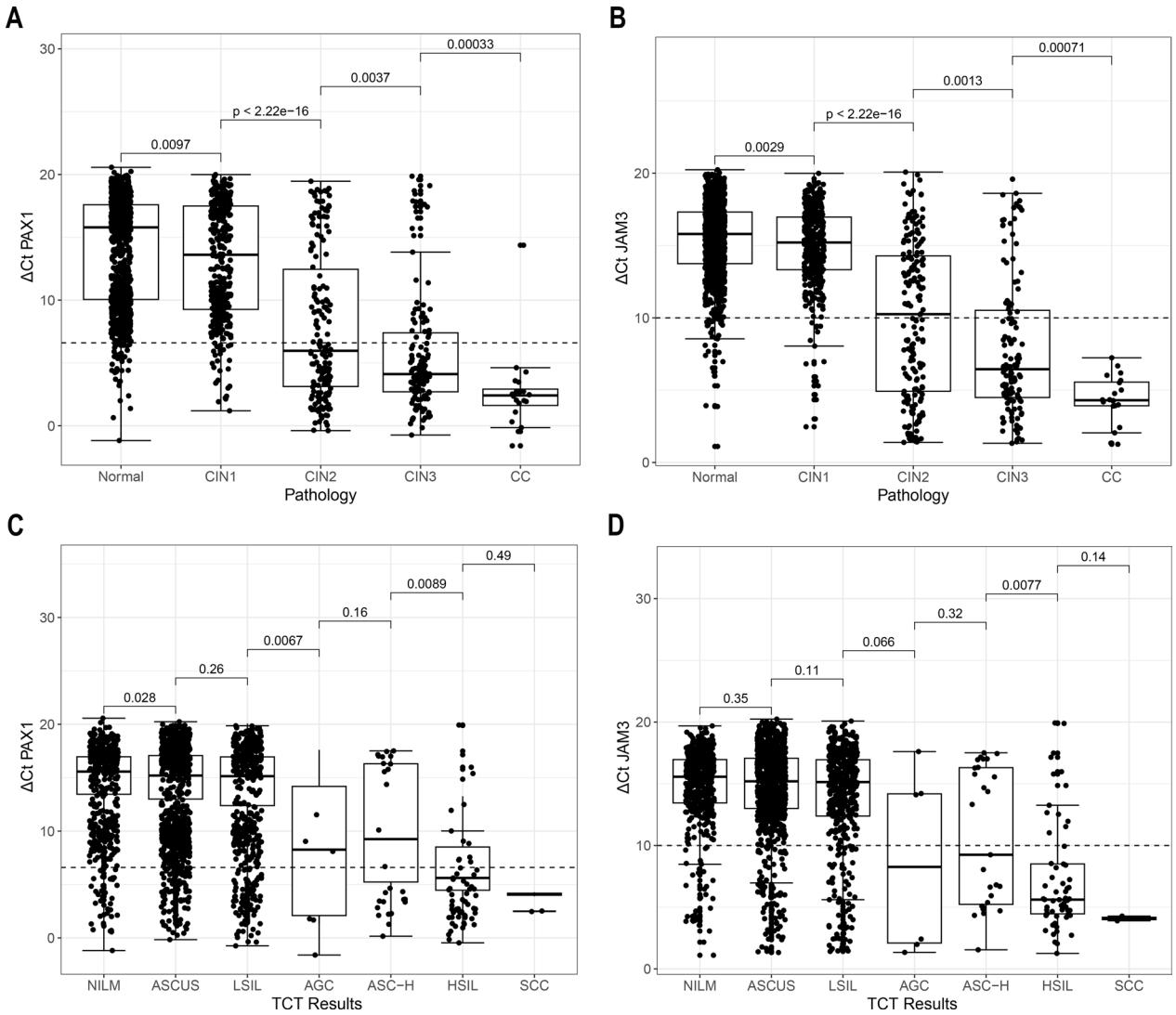

Fig. 2 *PAX1/JAM3* methylation degree (ΔCt) in the study group. **A** Distribution of the ΔCt of *PAX1* methylation on the basis of pathology results; **B** distribution of the ΔCt of *JAM3* methylation on the basis of pathology results; **C** distribution of the ΔCt of *PAX1* methylation on the basis of LBC results; **D** distribution of the ΔCt of *JAM3* methylation on the basis of LBC results. CIN, cervical intraepithelial neoplasia; CC, cervical cancer, including squamous cell carcinoma and adenocarcinoma; NILM, no intraepithelial lesions or malignancy; ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; AGC, atypical glandular cells; ASC-H, atypical squamous cells, cannot exclude HSIL; HSIL, high-grade squamous intraepithelial lesion

Performance of LBC ≥ ASCUS and *PAX1*^{m}/*JAM3*^{m} in detecting CIN3 +

Table 2 shows the diagnostic accuracy, sensitivity, specificity, and colposcopy referral percentages of various triage methods and their combinations across the whole cohort. Compared with that of *PAX1*^{m}(+) or JAM3^m(+) alone, the detection efficiency of *PAX1*^{m}/*JAM3*^{m} in combination for CIN3 + was improved in women who were not 16/18 hrHPV positive.

PAX1^m/*JAM3*^{m} had an AUC of 0.866 (0.837–0.896), which was higher than that of LBC ≥ ASCUS (AUC 0.584, 95% CI 0.558–0.610). *PAX1*^{m}/*JAM3*^{m} had a sensitivity of 84.8% (95% CI: 79.0%-90.5%) and a specificity of 88.5% (95% CI: 87.0%-90.0%).

Among all screening strategies, *PAX1*^{m}/*JAM3*^{m} triage resulted in the lowest colposcopy referral percentage (17.45%), which was significantly lower than the colposcopy referral percentage of LBC ≥ ASCUS (74.66%).

To investigate whether the combination of the two methods improves the detection of CIN3 +, we combined triage of *PAX1*^{m}/*JAM3*^{m} with LBC ≥ ASCUS, which led to an increased sensitivity of 98.0% (95% CI: 95.8–100%), but the specificity decreased to 24.8% (95% CI: 22.7–26.8%), and the referral percentage increased to 77.09%.

Risk of CIN3 + LBC ≥ ASCUS, and *PAX1*^{m}/*JAM3*^{m}(+) Results

The odds ratios (ORs) for CIN3 + in the various screening strategies are shown in Table 3. In the non-16/18 hrHPV(+) group, the odds ratio (OR) for CIN3 + in the *PAX1*^{m}/*JAM3*^{m}(+) subgroup compared with the *PAX1*^{m}/*JAM3*^{m}(-) subgroup was 42.6 (95% CI, 27.1–69.6). The OR for CIN3 + in the LBC ≥ ASCUS status subgroup

Table 3 The risks of CIN3 + in different triage methods

	Normal/CIN1/CIN2 n = 1700	≥ CIN3 n = 151	OR	*P* value
PAX1^m				< 0.001
(–)	1538 (90.5%)	37 (24.5%)	Reference	
(+)	162 (9.5%)	114 (75.5%)	29.1 (19.6–44.1)	
JAM3^m				< 0.001
(–)	1566 (92.1%)	35 (23.2%)	Reference	
(+)	134 (7.9%)	116 (76.8%)	38.4 (25.6–59.1)	
PAX1^m/*JAM3*^{m}				< 0.001
(–)	1505 (88.5%)	23 (15.2%)	Reference	
(+)	195 (11.5%)	128 (84.8%)	42.6 (27.1–69.6)	
LBC ≥ ASCUS				< 0.001
(–)	454 (26.7%)	15 (9.9%)	Reference	
(+)	1246 (73.3%)	136 (90.1%)	3.3 (2.0–5.9)	
LBC ≥ ASCUS or *P*^{m}/*J*^{m} (+)				< 0.001
(–)	421 (24.8%)	3 (2.0%)	Reference	
(+)	1279 (75.2%)	148 (98.0%)	15.4 (5.8–64.8)	
LBC ≥ ASCUS and *P*^{m}/*J*^{m} (+)				< 0.001
(–)	1538 (90.5%)	35 (23.2%)	Reference	
(+)	162 (9.5%)	116 (76.8%)	31.3 (20.9–47.8)	

CIN cervical intraepithelial neoplasia, *OR* odds ratio; *PAX1*^{m}(+): ΔCt *PAX1* ≤ 6.6; *JAM3*^{m}(+): ΔCt *JAM3* ≤ 10.0; *PAX1*^{m}/*JAM3*^{m}: ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS: liquid-based cytology test results atypical squamous cells of undetermined significance or worse; LBC ≥ ASCUS or *P*^{m}/*J*^{m}(+): liquid-based cytology test results atypical squamous cells of undetermined significance or worse or ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS and *P*^{m}/*J*^{m}(+): liquid-based cytology test results atypical squamous cells of undetermined significance or worse and ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0

Table 2 The detection performance of different triage methods for CIN3 + detection

Test methods	AUC (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Colposcopy referral cases n, % (95% CI)
PAX1^m(+)	0.830(0.795–0.865)	75.5(68.6–82.4)	90.5(89.1–91.9)	41.3(35.5–47.1)	97.7(96.9–98.4)	276, 14.91(13.36–16.61)
JAM3^m(+)	0.845(0.810–0.879)	76.8(70.1–83.6)	92.1(90.8–93.4)	46.4(40.2–52.6)	97.8(97.1–98.5)	250, 13.51(12.03–15.14)
PAX1^m/*JAM3*^{m}(+)	0.866(0.837–0.896)	84.8(79.0–90.5)	88.5(87.0–90.0)	39.6(34.3–45.0)	98.5(97.9–99.1)	323, 17.45(15.79–19.25)
LBC ≥ ASCUS	0.584(0.558–0.610)	90.1(85.3–94.8)	26.7(24.6–28.8)	9.8(8.3–11.4)	96.8(95.2–98.4)	1382, 74.66(72.63–76.59)
LBC ≥ ASCUS or *P*^{m}/*J*^{m} (+)	0.614(0.599–0.629)	98.0(95.8–100.0)	24.8(22.7–26.8)	10.4(8.8–12.0)	99.3(98.5–100.0)	1427, 77.09(75.12–78.95)
LBC ≥ ASCUS and *P*^{m}/*J*^{m} (+)	0.836(0.802–0.871)	76.8(70.1–83.6)	90.5(89.1–91.9)	41.7(35.9–47.5)	97.8(97.0–98.5)	278, 15.02(13.46–16.72)

AUC area under the curve, *PPV* positive predictive value, *NPV* negative predictive value; *PAX1*^{m}(+): ΔCt *PAX1* ≤ 6.6; *JAM3*^{m}(+): ΔCt *JAM3* ≤ 10.0; *PAX1*^{m}/*JAM3*^{m}: ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS: liquid-based cytology test results atypical squamous cells of undetermined significance or worse; LBC ≥ ASCUS or *P*^{m}/*J*^{m}(+): liquid-based cytology test results atypical squamous cells of undetermined significance or worse or ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; LBC ≥ ASCUS and *P*^{m}/*J*^{m}(+):liquid-based cytology test results atypical squamous cells of undetermined significance or worse and ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM 3* ≤ 10

compared with the NILM status subgroup was 3.3 (95% CI, 2.0–5.9).

Discussion

In this study, we investigated the clinical utility of the *PAX1*^{m}*/JAM3*^{m} assay as a triage test for non-16/18 hrHPV(+) patients in routine cervical screening. The ΔCt values of *PAX1* and *JAM3* methylation decreased as histopathology severity increased. Furthermore, in this study, the triage capabilities of various detection methods for cervical cancer screening in non-16/18 hrHPV(+) patients were comprehensively evaluated. These results indicate that the use of *PAX1*^{m}*/JAM3*^{m} from cervical epithelial cells is highly accurate in detecting CIN3 + lesions with a sensitivity that is similar to that of LBC tests but with significantly higher specificity, thus greatly reducing colposcopy referrals.

The prevalence of high-risk HPV types varies geographically. In parts of Asia, HPV31/33/52/58 are reported as frequently as HPV16 or HPV18 in precancerous lesions [37]. According to a study in southern Shanghai, China, in terms of the total proportion of hrHPV infections, there were more non-16/18 hrHPV infections (68.22%) than HPV16/18 hrHPV infections. Non-16/18 hrHPV infection accounts for 50.84% of patients with HSILs or cervical cancer (HSILs +) [38]. On the basis of the results of this study from screening hospital-based gynecology clinics, we found that the prevalence of non-16/18 infections was 15.7% (4735/30084) of the total screened population. Because non-16/18 hrHPV types are associated with a higher prevalence of infection, the triage of non-16/18-positive women may be more effective in identifying high-risk patients. Moreover, as non-16/18 hrHPV infections account for a higher proportion of patients with HSILs or cervical cancer, there is a greater need for an effective management strategy that identifies and triages high-risk women so that preventive measures can be developed and implemented to reduce the incidence of these types of HPV-associated cervical cancers. Cytology, an option for the triage of hrHPV(+) women, not only results in an increased burden of care due to increased colposcopy referrals but also may result in unnecessary overdiagnosis [11, 12]. In this study, cytology demonstrated low specificity, potentially influenced by the designated positive threshold. In this study, we aimed to evaluate various detection methods for identifying CIN3 + patients. The elevated positivity rates of CIN1 and CIN2 by cytology are shown in Table 1. This contributes to the reduced specificity of cytology for detecting CIN3 +. These findings suggest that cytology may not be an effective triage tool and that it is limited in accurately identifying high-risk individuals among non-16/18 HPV-positive women. Another limitation of cytological analysis in the triage of non-16/18 hrHPV(+) patients is that despite the 90.1% sensitivity of LBC for the detection of CIN3 +, there were still four cases of cervical cancer that were not detected by cytology. These patients were asked to undergo colposcopy for further evaluation because of abnormal vaginal bleeding, suggesting that in some cases, liquid-based cytology may miss some cases. In contrast, methylation testing was capable of identifying all the cervical cancer samples.

In this study, the diagnostic accuracy of *PAX1*^{m}*/JAM3*^{m} was similar to that of previously reported methylation tests [39]. Previous studies on the application of methylation for triage in hrHPV(+) patients have shown that methylation has a diagnostic ability equal to or even superior to that of cytological pathology. As reported, the CIN3 + sensitivity of *FAM19A4/miR124-2* methylation analysis was similar to that of cytology (71.3% vs. 76.0%), with a lower specificity (78.3% vs. 87.0%) [40]. E6/E7 detection combined with *PAX1* methylation is superior to HPV testing for cervical cancer screening, and the AUC of *PAX1* (0.919) is significantly higher than those of HPV (0.541) and E6/E7 detection (0.607) ($p < 0.0001$) [41]. *CADM1/MAL* methylation in combination with cytology triage of hrHPV(+) patients was better than cytology alone, and combined triage revealed a sensitivity of 86.8% vs. 65.8% for CIN3(+) and a corresponding specificity of 64.8% versus 78.6% [42]. To investigate whether combining LBC and *PAX1*^{m}*/JAM3*^{m} testing improves the efficacy of CIN3 + detection. We performed a comparison and found that the combination of the two methods did not enhance the detection efficacy for CIN3 +. In other words, the triage of *PAX1*^{m}*/JAM3*^{m} alone was not only superior to cytology testing alone but also preferable to a combined program of both methylation and cytology (*PAX1*^{m}*/JAM3*^{m}(+) vs. LBC ≥ ASCUS(+) vs. LBC ≥ ASCUS and/or *PAX1*^{m}*/JAM3*^{m}(+), AUC: 0.866 vs. 0.584 vs. 0.836/0.614). The combined LBC ≥ ASCUS and *PAX1*^{m}*/JAM3*^{m}(+) triage revealed a CIN3(+) sensitivity of 98.0%, which was greater than the 90.1% for LBC ≥ ASCUS and the 84.8% for *PAX1*^{m}*/JAM3*^{m}(+). Recent studies have shown that the DNA methylation levels of host cell genes and viral genes increase with the severity of CIN and are very high in cervical cancer patients [43–46]. These findings suggest that methylation testing may be more effective in diagnosing more severe cervical lesions. This suggests that methylation testing could be more effective in diagnosing more severe cervical lesions, whereas cytology might fail to be as useful. This finding also suggests that morphological reliability is still not a substitute for the accuracy of molecular markers and that differences in methylation levels between cytologic types do not account for differences in histopathology.

As a triage method for non-16/18-positive women, the application prospects of $PAX1^m/JAM3^m$ are promising. Compared with LBC ≥ ASCUS in this study, $PAX1^m/JAM3^m$ was more effective in reducing colposcopy referrals (17.45% vs. 74.66%). These findings are similar to the results of a previous study of methylation for hrHPV triage [32], where both $PAX1^m$ and $ZNF582^m$ reduced colposcopy referrals when LBC ≥ ASCUS were compared (30.2%, 26.9% vs. 50.7%, respectively). Our study revealed a significant odds ratio for ≥ CIN 3 + for LBC ≥ ASCUS and $PAX1^m/JAM3^m$. These results suggest that among non-16/18 hrHPV(+) women, $PAX1^m/JAM3^m$(+) patients are more likely to need direct colposcopy than follow-up.

This study also has certain limitations. First, the sample sizes were uneven across centers. Second, evaluations and considerations of the timing and extent of high-risk human papillomavirus (hrHPV) infection were lacking in our study. No patients were followed up, and the risk of short- or long-term progression could not be distinguished between patients with LBC ≥ ASCUS and patients with $PAX1^m/JAM3^m$. Future prospective studies with large sample sizes in different regions and adequate follow-up data are needed to confirm our findings.

Conclusion

In non-16/18 hrHPV(+) women, $PAX1^m/JAM3^m$ was superior to cytology for detecting CIN3 + with favorable diagnostic sensitivity and specificity and significantly reduced referrals to colposcopy. The combination of $PAX1^m/JAM3^m$ and cytology was not more advantageous than $PAX1^m/JAM3^m$ alone for triage.

Supplementary Information

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Additional file 1. The demographic characteristics and clinical information for each center.

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None

Author contributions
LL, GX, YH, SL, JZ, and HS conceived of the original idea for the study, interpreted the results, carried out the statistical analysis, edited the paper and were overall guarantors. XC, XJ, ZD, SZ, BQ, JF, and XC obtained ethical approval, contributed to the preparation of the dataset, interpreted the results and contributed to drafts of the paper. YL and PL contributed to the study design and interpretation of the results, and commented on drafts of the paper. LK conducted the pathological evaluation and reviewed the original materials. All the authors have approved the final version of the manuscript.

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Availability of data and material
No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate
The Institutional Review Board of Peking Union Medical College Hospital approved this study (No. JS-2380 and K-S2021211 for METHY3 and METHY4, respectively). The registration numbers are NCT04646954 and NCT05290428 (*clinicaltrials.gov*, registered on November 26, 2020, and March 12, 2022). Informed consent was obtained from the subjects prior to their participation in the study.

Statement of submission
The paper is not under consideration by another journal, and the results presented in this work have not been previously presented or published.

Consent for publication
Consent for publication has been obtained from all patients.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Beijing 100730, China. [2]National Clinical Research Center for Obstetric & Gynecologic Diseases, Beijing 100730, China. [3]State Key Laboratory for Complex, Severe and Rare Diseases, Peking Union Medical College Hospital, Beijing 100730, China. [4]Department of Medical Laboratory, Beijing Origin-Poly Bio-Tec Co., Ltd., Beijing 102600, China. [5]Clinical Precision Medicine Research Center, the First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou 510062, China. [6]Department of Obstetrics and Gynecology, Peking University International Hospital, Beijing 100000, China. [7]Department of Obstetrics and Gynecology, Inner Mongolia Autonomous Region People's Hospital, Hohhot 010000, China. [8]Department of Gynecology, Cangzhou Central Hospital, Cangzhou 061000, China. [9]Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310000, China. [10]Department of Obstetrics and Gynecology, Zhejiang Provincial People's Hospital, Hangzhou 310000, China.

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