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Cervical cancer screening: efficacy of *PAX1* and *JAM3* methylation assay in the triage of atypical squamous cell of undetermined significance (ASC-US)

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Abstract

Background Atypical squamous cells of undetermined significance (ASC-US) often present diagnostic challenges with cytology-based results, leading to potential underdiagnosis or overdiagnosis. An effective triage method is essential for managing these cases to reduce unnecessary referrals and treatment.

Methods A total of 322 women diagnosed with ASC-US were tested for HPV-DNA and the *PAX1* and *JAM3* methylation ($PAX1^m$/$JAM3^m$) test in the study.

Results Methylation levels of *PAX1* and *JAM3* were significantly elevated in cervical lesions classified as CIN2 or more severe lesions (CIN2+). The methylation assay demonstrated a sensitivity of 83.8% and a specificity of 95.8%, outperforming HPV-DNA testing in differentiating high-grade cervical lesions among women with ASC-US. Moreover, $PAX1^m$/$JAM3^m$ testing significantly reduced the colposcopy referral rate for further diagnostic procedures in high-risk HPV-positive women by 79.5%.

Conclusions $PAX1^m$/$JAM3^m$ testing shows promise as a reliable supplemental method to HPV-DNA testing for the triage of women with cytologic ASC-US. In addition, the molecular triage based on the CISCER assay or single *PAX1* or *JAM3* methylation, had better effects in the women with non-HPV16/18 group. This approach could potentially minimize overtreatment and unnecessary referrals in clinical practice, enhancing patient management and resource utilization.

Keywords Cervical cancer, ASC-US, Methylation, *PAX1*, *JAM3*

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Introduction

The cancer statistics for 2022 reveal that the morbidity and mortality rates for cervical cancer remain high, with approximately 660,000 new cases and nearly 350,000 deaths [1]. Cervical intraepithelial neoplasia (CIN), the precursor to cervical cancer, progresses through three stages—CIN1, CIN2, and CIN3—with increasing severity. The long period of progression offers multiple opportunities for intervention before the lesions advance to invasive cancer [2]. Therefore, effective screening and timely treatment for precancerous lesions are key strategies for alleviating the burden of cervical cancer [3–5].

For decades, Pap smear cytology has played a crucial role in reducing the incidence and mortality of cervical cancer in developed countries with established cervical screening programs [6]. However, the burden remains high in developing and underdeveloped countries and areas. The World Health Organization (WHO) recommends HPV-DNA testing as the primary screening method due to its high sensitivity and superior preventative outcomes, and many countries have adopted this approach [7]. Despite this, over 80% of women will exhibit cervical HPV infections during their lifetime, with around 90% of these infections being cleared naturally [8]. Persistent infection with high-risk HPV is correlated with an elevated risk of developing high-grade CIN and progression to cervical cancer. Consequently, liquid-based cytology (LBC) is a widely accepted triage method for HPV-positive women, and co-testing with both cytology and HPV tests is recommended where resources allow [9, 10].

However, cytology also has notable shortcomings in cervical intraepithelial neoplasia (CIN) detection, such as low sensitivity and poor reproducibility of results that depend on pathologists' interpretation [11, 12]. Moreover, for low-grade cytological results (atypical squamous cells of undetermined significance, ASC-US, and low-grade squamous intraepithelial lesions, LSIL), the specificity for detecting CIN2+is considerably lower. According to The Chinese Society for Colposcopy and Cervical Pathology of China Healthy Birth Science Association (CSCCP), ASC-US constitutes over 50% of cytological abnormalities in China, while the rate of high-grade lesions of pathology among ASC-US cases is usually less than 30% [13–16]. Given the multiple causes of ASC-US results, it presents a challenging issue in clinical management to effectively avoid under- or over-diagnosis. Although excluding women with HPV-negative among ASC-US can help reduce the colposcopy referral rate, the reduction is limited [11, 14]. Thus, referring all women with ASC-US to colposcopy would result in unnecessary procedures. Overall, there is a critical need for developing more innovative and reliable biomarkers to assist in the triage of ASC-US cases in China.

Epigenetic modifications are closely linked to carcinogenesis, progression, and metastasis in various cancer types [17]. Recent studies have identified abnormal DNA methylation in genes such as *SOX1* [18], *PAX1* [19], *FAM19A4*/*miR124-2* [20], *C13ORF18/JAM3/ANKRD18CP* [21], *JAM3*/*EPB41L3* [22], *ZNF671* [23] as promising biomarkers for detecting cervical (pre)cancer. Among these, *PAX1*, a member of the paired box (PAX) transcription factors family, plays a significant role in many biological processes [24]. Elevated *PAX1* methylation levels have been observed in numerous cancer-related cells [25–28]. Mechanically, *PAX1*, *WDR5*, and *SET1B* form a complex that enhances trimethylation of histone H3 at lysine 4, activating multiple phosphatases (*DUSP5*, *PTPRR*, *DUSP1*, *DUSP6*) in cervical cancer cells [27], thereby regulating phosphatase-kinase balance in cervical epithelium. Additionally, *PAX1* methylation also correlates with radio-resistance in cervical cancer [29]. *JAM3*, part of the junctional adhesion molecule protein family, is epigenetically silenced in cancer patients, which can promote tumor progression and affecting prognosis [30–32]. In cervical cancer, hypermethylation leads to decreased *JAM3* expression in 52.3% of cervical squamous cell carcinoma cases [33]. The methylation panel combining the *PAX1* and *JAM3* genes has shown promising potential in cervical screening [34–36].

In the present study, we assessed the triage effectiveness of *PAX1* and *JAM3* methylation ($PAX1^m$/$JAM3^m$) detection among women with cytological ASC-US results. Our goal is to provide a novel molecular triage strategy for these women, with the aim of enhancing clinical management and facilitating more effective interventions for cervical cancer.

Materials and methods

Participants and study design

This cross-sectional study enrolled women who underwent both HPV and cytology testing during outpatient opportunistic screenings at Zhejiang Provincial People's Hospital, from March 2022 to January 2024. It is approved by the Ethics Committee of Zhejiang Provincial People's Hospital (No.2021SJ020), and which strictly follows the ethical norms of institutions and the National Research Council regarding human subject research. All participants have approved the written informed consent for this study. Only those with cytological ASC-US were included for the study (Fig. 1). The inclusion criteria were as follows: age 18 or older, sexual experience, and sign informed consent to participate in this study. The exclusion criteria included the following: a history of cervical surgery, a history of malignant tumors, HIV infection, immunological disorders, prior organ transplantation, pregnancy, current treatment for malignant tumors, or

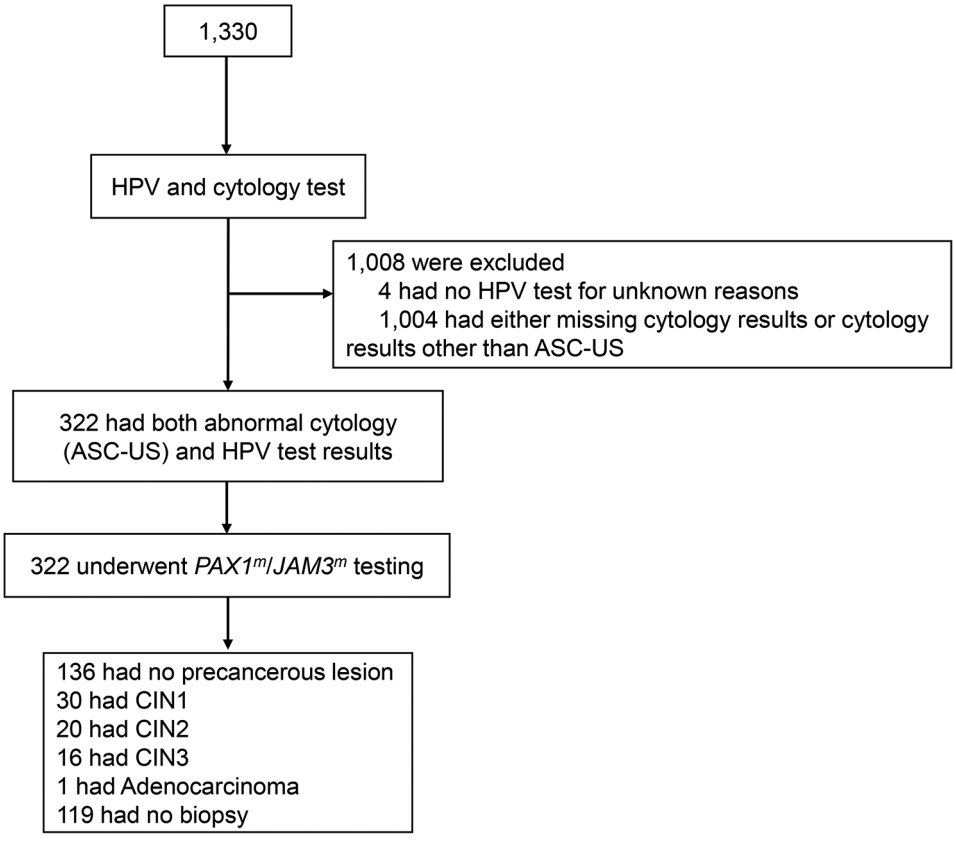

Fig. 1 The flow chart of this study

the use of immunosuppressive medications within the past six months.

Sample collection and research procedures

This study is conducted as the following process: Cervical exfoliated cell samples were collected using Cervex Brush (Rovers Medical Devices, North Holland, Netherland) and stored in Thinprep PreservCyt solution (Hologic, MA, USA) for liquid-based cytological examination, HPV-DNA testing, and methylation detection. The HPV-DNA testing was performed using the Cobas 4800 HPV Controls Kit (Roche, Shanghai, China) in accordance with the manufacturer's guidelines. The results included the identification of individual HPV types 16 and 18, along with twelve high-risk HPV (hrHPV) types, referred to as non-HPV16/18 hrHPV: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

The cytological testing results were evaluated by 1–2 qualified physicians, in accordance to the Bethesda system (the newest version updated in 2014 [37]). Women with cytological ASC-US findings, regardless of hrHPV status (positive or negative), will be referred for a colposcopic examination. During colposcopy, visual examination of the cervix was performed using acetic acid and/or Lugol's iodine solution, and colposcopy-guided biopsies were conducted for abnormal lesions. Endocervical curettage (ECC) will be performed on women with a transformation zone type 3. For women with normal colposcopy findings, the decision of whether to perform no biopsy or one to two cervical biopsies is based on the physician's clinical experience and the patient's consent. If CIN or cervical cancer was diagnosis, the women would be recommended treatment in accordance with the Chinese Guidelines for Diagnosis and Treatment of Cervical Cancer (2023). The histological classification of specimens was assessed by 2–3 qualified pathologists in accordance with international standards. This classification includes normal (no CIN findings), CIN1, CIN2, CIN3, and invasive cervical cancer. The most severe histological finding from cervical biopsy, endocervical curettage (ECC), or surgery was considered the final pathology result for analysis.

DNA extraction and isolation,and methylation analysis

The residual cervical exfoliated cell samples in Thinprep PreservCyt solution were used for methylation detection, which is conducted in a certified DNA laboratory. The study staff and methylation testing operators were kept blinded to the clinical information, cytology, HPV genotyping, and cervical histopathology results for these

cases. Genomic DNA (gDNA) was extracted from the exfoliated cervical sample using the JH-DNA Isolation and Purifying kit (OriginPoly Bio-Tec Co., Ltd., Beijing, China) with the reference to the manufacturer's instructions. The DNA concentration is quantified by using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, DE, USA). Subsequently, 200–1000 ng of gDNA is applied to bisulfite conversion using JH-DNA Methylation-Lightning MagPrep (OriginPoly Bio-Tec Co., Ltd., Beijing, China). The methylation levels of *PAX1* and *JAM3* are detected using the Human *PAX1* and *JAM3* Methylation Detection Kit (Real-time PCR), also known as CISCER (OriginPoly Bio-Tec Co., Ltd., Beijing, China) by SLAN-96 S automatic medical PCR analysis system (Shanghai Hongshi Med Tech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been designated as the internal control within the kit. This kit is classified as a medical device class III approved by the China National Medical Products Administration (No. 20233400253). Briefly, the PCR reactions began with an initial incubation at 96 °C for 10 min in a single cycle, followed by 45 cycles of denaturation at 94 °C for 15 s, annealing at 64 °C for 5 s, and extension at 60 °C for 30 s. Finally, the reaction was cooled to 25 °C for 1 min. The hypermethylation level of *PAX1* and *JAM3* genes are determined by the difference between the two Ct values (ΔCt_{PAX1}=Ct_{PAX1}-Ct_{GAPDH} and ΔCt_{JAM3}=Ct_{JAM3}-Ct_{GAPDH}). Per the manufacturer's instructions, the positivity of the $PAX1^m$/$JAM3^m$ test is defined as $\Delta Ct_{PAX1} \leq 6.6$ and $\Delta Ct_{JAM3} \leq 10.0$, respectively.

Table 1 Baseline characteristics of the study population with atypical squamous cells of undetermined significance (ASC-US)

Characteristic	Total (*N* = 322)
Results of histology	
No CIN	136 (42.2%)
CIN1	30 (9.3%)
CIN2	20 (6.2%)
CIN3	16 (5.0%)
Adenocarcinoma	1 (0.3%)
No histological results	119 (37.0%)
HPV results	
Positive	286 (88.8%)
HPV-16/18	80 (24.8%)
Non-16/18 hrHPV	206 (64.0%)
Negative	36 (11.2%)
Age, median (range), y	40.0 [20, 75]
Age group, y	
< 30	47 (14.6%)
30–39	111 (34.5%)
40–49	63 (19.5%)
≥ 50	101 (31.4%)
Δ Ct*PAX1*	
Mean (SD)	12.8 (4.9)
Median [Min, Max]	13.9 [0.7, 19.7]
Δ Ct*JAM3*	
Mean (SD)	14.2 (3.6)
Median [Min, Max]	14.9 [2.4, 19.7]
CISCER	
Negative	275 (85.4%)
Positive	47 (14.6%)

Data represent no. (%) of study participants unless otherwise specified. HPV-16/18: HPV16 and (or) HPV18 types. Non-16/18 hrHPV: HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. No CIN means no CIN found during colposcopy examination. CISCER positive Δ*Ct*$_{PAX1} \leq 6.6$ and (or) Δ*Ct*$_{JAM3} \leq 10.0$; CISCER negative criteria: Δ*Ct*$_{PAX1} > 6.6$ and Δ*Ct*$_{JAM3} > 10.0$

Abbreviations: SD, Standard Deviation; N, number; Min, minimum value; Max, maximum value; CIN, cervical intraepithelial neoplasia; CIN1, CIN2, and CIN3, IN grade 1, 2, and 3; HPV, human papillomavirus; hrHPV, high-risk HPV; *PAX1*, paired box 1; *JAM3*, junctional adhesion molecule protein 3

Statistical analysis

All statistical analyses were conducted using R version 4.2.1 (2022-06-23). Receiver operating characteristic curves (ROC), areas under the curve (AUC), and corresponding 95% confidence intervals were generated using the pROC package, version 1.18.0. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), along with their 95% confidence intervals, were calculated using the epi.tests and BDtest functions in the epiR (version 2.0.38) and bdpv (version 1.3) packages, respectively. Categorical variables were presented as numbers with percentages, and continuous variables as medians with interquartile ranges (Q1-Q3) or means with standard deviations. The Wilcoxon rank-sum test was used to compare continuous variables between groups, while the chi-square test or Fisher's exact test was applied for categorical variables. A p value of 0.05 was considered statistically significant.

Results

Baseline and molecular characteristics

A total of 322 women with cytological ASC-US who were referred for colposcopy participated in this study. Their baseline clinical and molecular characteristics are summarized in Table 1. The cohort has a median age of 40 years, ranging from 20 to 75 years old. Of these cases, 80 tested positive for HPV 16 /18, 206 for non-16/18 hrHPV, and 36 were found to be negative for hrHPV. The histological analysis identified a total of 203 cases, including 136 cases with no evidence of CIN, 30 cases classified as CIN1, 20 cases categorized as CIN2, 16 cases designated as CIN3, and one case diagnosed as adenocarcinoma.

We categorized the 203 cases who had histological results into two groups: low-grade lesions (CIN1-, including no lesions found and CIN1) and high-grade lesions (CIN2+, including CIN2, CIN3, and cancer) (Table 2). Among these, all 18 hrHPV-negative women had CIN1- lesions, and their CISCER assay results were all negative. For HPV 16/18-positive women, 68.0%

(34/50) had CIN1- lesions, while the proportion reached 84.4% (114/135) in non-16/18 hrHPV-positive women. Of the HPV 16/18-positive women with CIN1- lesions, 94.1% (32/34) tested negative for methylation, while 95.6% (109/114) of non-16/18 high-risk HPV-positive women with CIN1- lesions also had negative methylation results. Among the 37 women with CIN2+lesions, 16 cases (43.2%) were infected with HPV 16/18, while the remaining were infected with non-16/18 hrHPV. Additionally, 31 cases (83.8%) tested positive for methylation assay (Table 2 & Supplementary Table 1).

The characteristics of patients without histological results are presented in Supplementary Table 2. Total positive CISCER was 7.6% in cases without histological results, while it was 8.9% (9/101) in cases with positive hrHPV results. In addition, among the 18 women with negative hrHPV results, CISCER assay results were also negative.

Evaluation of *PAX1* and *JAM3* genes methylation level

The methylation levels of *PAX1* and *JAM3* genes were compared among women with no CIN, CIN1, CIN2, CIN3, and cancer (Fig. 2A and B). The ΔCt values for both genes decreased markedly from the CIN1 group to the CIN2 group ($p<0.001$), and remained low in the CIN3 group. The adenocarcinoma case exhibited extremely low ΔCt values for both *PAX1* (1.78) and *JAM3* (3.4). This indicates that the methylation levels of both genes were significantly higher (with lower ΔCt values) in women with high grade lesions compared to those with low-grade lesions. In women with no CIN or CIN1, the positive methylation for either *PAX1* or *JAM3* was 2.4% (4/166), while the positivity for both markers was recorded at 1.8% (3 out of 166), respectively.

In CIN2, 30.0% (6/20) were positive for one gene, while 50.0% (10/20) were positive for both. For CIN3, 37.5% (6/16) showed positivity in one gene, and 50.0% (8/16) in both genes (Fig. 2C).

Triage performance of *PAX1*/*JAM3* gene methylation

The triage value of $PAX1^m/JAM3^m$ testing for ASC-US was further evaluated (Table 3). For CIN2+, $PAX1^m$ demonstrated a sensitivity of 70.3% (55.5–85.0%), and $JAM3^m$ presented a sensitivity of 64.8% (49.5–80.3%). Combining both genes ($PAX1^m$ or $JAM3^m$, CISCER assay) increased sensitivity to 83.8% (71.9–95.7%) with high specificity of 95.8% (92.7–98.8%), comparable to the specificity of individual gene: $PAX1^m$ at 97.6% (95.3–99.9%) and $JAM3^m$ at 96.4% (93.5–99.2%). The PPV of single $PAX1^m$, $JAM3^m$, and CISCER assay were 86.7% (74.5–98.8%), 80% (65.7–94.3%), and 81.6% (69.3–93.9%), respectively. The NPV for these were 93.6% (90.0-97.3%), 92.5% (88.6–96.4%), and 96.4% (93.5–99.2%), respectively. The methylation analysis of both individual and combined genes demonstrates that the odds ratios and ROC curves are significantly elevated, resulting in a lower colposcopy referrals rate compared to HPV testing (see Table 3; Fig. 3).

CISCER assay showed a relative sensitivity of 0.84 (0.72–0.96) compared to HPV testing, while its relative specificity was notably high at 8.84 (6.35–15.18) (Fig. 4). This indicates that, under comparable sensitivity conditions, the specificity of the CISCER test is 8 times that of the HPV test. For women with histological findings in this study, the referral rate for colposcopy among hrHPV-positive is 91.1% which represented a mere reduction of 8.9% with no CIN2+lesions missed. However, using CISCER assay for triage could lower the referral rate by 81.3%. While CISCER assay might miss some CIN2+lesions, most of these are in younger women, including 4 CIN2 cases (all under 40 years old) and 2 CIN3 cases (one under 30 years old and one over 50 years old). The immediate CIN3+risk based on negative CISCER assay results was 1.2% (2/165), compared to 39.5% (15/38) based on positive CISCER assay results. If the 18 hrHPV-negative women who did not undergo biopsy were considered to have no high-grade lesions, the estimated referral rate would be reduced by 16.3% (36/221) with HPV testing and by 82.8% (183/221) with CISCER assay. Furthermore, when CISCER assay was

Table 2 Characteristics of women with histological results

	Total ($n=203$)	**CIN1- ($n=166$)**		**CIN2+ ($n=37$)**	
Age Median(Q1,Q3)	39.0 (32.0, 52.3)	39.0 (33.0, 52.8)		37.0 (31.0, 50.5)	
		CISCER Positive	**CISCER Negative**	**CISCER Positive**	**CISCER Negative**
hrHPV Positive	185(91.1%)	7(4.2%)	141(84.9%)	31(83.8%)	6(16.2%)
HPV-16/18	50(27.0%)	2	32	12	4
Non-16/18 hrHPV	135(73.0%)	5	109	19	2
hrHPV Negative	18(8.9%)	0(0%)	18(10.8%)	0(0%)	0(0%)

CIN1-: No CIN or CIN grade 1; CIN2+: CIN grade 2 or higher lesions. Data represent no. (%) of study participants unless otherwise specified. HPV-16/18: HPV16 and (or) HPV18 types. Non-16/18 hrHPV: HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. CISCER positive ΔCtPAX1 ≤ 6.6 and (or) ΔCtJAM3 ≤ 10.0; CISCER negative criteria: ΔCtPAX1 > 6.6 andΔCtJAM3 > 10.0

Abbreviations: n, number; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; hrHPV, high-risk HPV

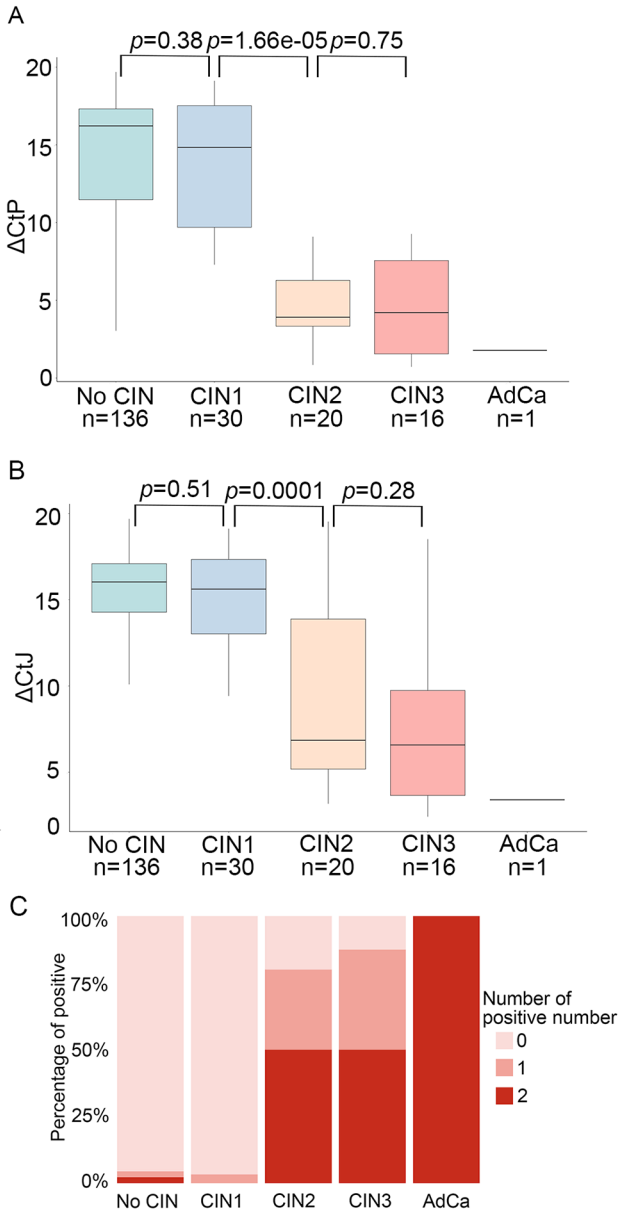

Fig. 2 Methylation levels of *PAX1* (**A**) and *JAM3* (**B**) genes in different grade of lesions, and positive rates of *PAX1* and/or *JAM3* methylation in cases with no CIN, CIN1, CIN2, CIN3, and AdCa (**C**). CIN: cervical intraepithelial neoplasia. AdCa, adenocarcinoma

implemented to further triage ASC-US women who tested hrHPV-positive, it effectively reduced the referral rate by 79.5% (147/185).

Efficacy of *PAX1*/*JAM3* gene methylation in triaging women infected with HPV16/18 and non-16/18 hrHPV

The *PAX1*/*JAM3* gene methylation triage testing for CIN2+was further investigated among ASC-US cases with HPV16/18 and non-16/18 hrHPV infection (Supplementary Table 3). In the HPV16/18-positive of ASC-US group, the sensitivity of $PAX1^m$, $JAM3^m$, and CISCER assay were 62.5% (46.0-91.5%), 50.0% (31.9–80.6%), 75.0% (53.8–96.2%), respectively, while their specificities were 97.1% (94.3–100%), 94.1% (90.9–100%), and 94.1% (90.9–100%). In contrast, the non-16/18 hrHPV if ASC-US group showed superior sensitivities, with $PAX1^m$ and $JAM3^m$ at 71.4% (52.1–90.8%) and CISCER assay at 90.5% (77.9–100%). Specificities were also higher in this group, with $PAX1^m$ at 97.4% (95.2–100%), $JAM3^m$ at 96.5% (94.1–99.9%), and CISCER assay at 95.6% (93.0-99.5%). These findings suggest that molecular triage using CISCER assay may be more effective in the non-HPV16/18 group.

Discussions

The interpretation of ASC-US results presents significant clinical decision-making challenges. The ASCCP guidance recommends timely detection and treatment for the highest-grade precancerous lesions and cancer (CIN 3+) due to their higher immediate and 5-year risk [10]. Only 5.3% (17/203) of CIN3+cases were identified in ASC-US, which often leads to excessive anxiety and overtreatment due to the limited specificity observed in this study. This relatively low proportion again suggests that the risk of high-grade cervical pre-cancerous lesions in women with ASC-US cytology is not high. Additionally, the abnormalities associated with the combination of hrHPV and ASC-US testing did not lead to a reduction in the number of colposcopy referrals. This is mainly due to the higher proportion of ASC-US in cervical cytology in China. CISCER assay exhibited high sensitivity (83.8%) and specificity (95.8%) for CIN2+detection in this study, with an AUC of 0.898. Our findings highlighted the clinical potential of CISCER assay as a complementary method to HPV testing for improving management of the ASC-US patients.

The WHO Guidelines for Screening and Treatment of Cervical Precancerous Lesions (Second Edition), published in 2021, noted the potential of methylation testing for future cervical cancer screening [38]. Previously, researchers have proposed that host gene methylation testing could help address overdiagnosis and overtreatment issues and serve as an option for ASC-US triage testing. This approach, however, must demonstrate that test-negative women have a low risk of cancer in the triage process [39]. Earlier research indicated that CCNA1 promoter methylation had a sensitivity of 19.0% for detecting CIN2+lesions in the ASC-US population in Thailand, though it had a specificity of 99.3% [40]. In contrast, CISCER assay demonstrated a much higher sensitivity and effectively distinguished between CIN1- and CIN2+lesions. Although this study noted false-negative results for CIN2+, as reported by others, the risk of CIN3+among CISCER-negative individuals was found to be only 1.2%, with no cases of missed cancer observed.

Table 3 Performance of screening tests for the detection of cervical intraepithelial neoplasia of Grade 2 or higher

	Accuracy, No. Correct/ Total No. (%)								
	No CIN	CIN1	CIN2+	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV	NPV	OR	Referral rate, %
1. PAX1	132/136 (97.1)	30/30 (100)	26/37 (70.3)	70.3 (55.5–85.0)	97.6 (95.3–99.9)	86.7 (74.5–98.8)	93.6 (90.0-97.3)	95.7	14.8
2. JAM3	131/136 (96.3)	29/30 (96.7)	24/37 (64.9)	64.8 (49.5–80.3)	96.4 (93.5–99.2)	80 (65.7–94.3)	92.5 (88.6–96.4)	51.3	14.8
3. CISCER	130/136 (95.6)	29/30 (96.7)	31/37 (83.8)	83.8 (71.9–95.7)	95.8 (92.7–98.8)	81.6 (69.3–93.9)	96.4 (93.5–99.2)	121.1	18.7
4. HPV	17/136 (12.5)	1/30 (3.3)	37/37 (100)	100 (100–100)	10.8 (6.1–15.6)	20 (14.2–25.7)	100 (100–100)	/	91.1

Note: Referral rate (based on the percentage of test positivity). CIN2+: CIN grade 2 or higher severity

Data represent no. (%) of study participants unless otherwise specified. HPV: HPV 16,18,31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. No CIN means no CIN found during colposcopy examination. CISCER positive ΔCt*PAX1* ≤ 6.6 and (or) ΔCt*JAM3* ≤ 10.0; CISCER negative criteria: ΔCt*PAX1* > 6.6 and ΔCt*JAM3* > 10.0

Abbreviations: No., number; CIN, cervical intraepithelial neoplasia; CIN1, CIN2, and CIN3, IN grade 1, 2, and 3; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; HPV, human papillomavirus; *PAX1*, paired box 1; *JAM3*, junctional adhesion molecule protein 3

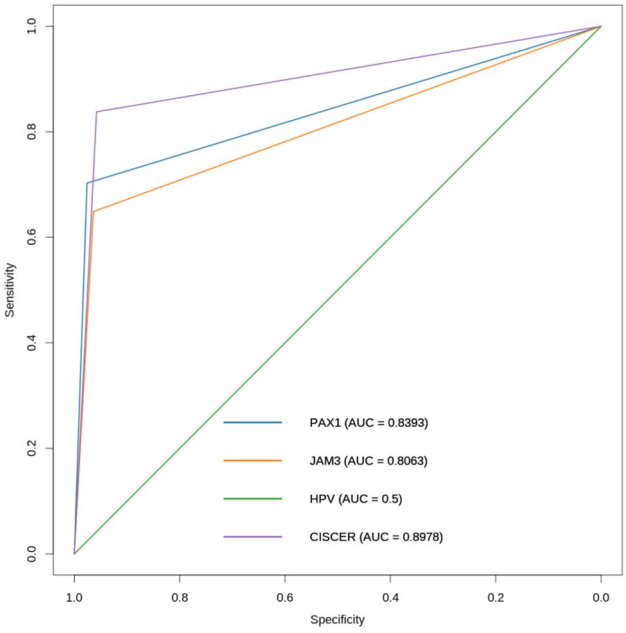

Fig. 3 ROC of different triage methods for ASC-US patients with CIN2+. AUC: area under the curve

Among the missed cases, most were diagnosed with CIN2 lesions in young women. As we know, CIN2 lesions often regress spontaneously in young women and have a low risk of progressing to cervical cancer [41, 42]. Consequently, current clinical management strategies generally recommend follow-up rather than immediate treatment to protect the cervix and minimize potential health risks associated with overtreatment in this population. CIN3 lesions also have the possibility of regression [43, 44]. Their progression to cancer, linked to the accumulation of epigenetic alterations, has been documented [39]. Whether the women with low *PAX1* and *JAM3* methylation levels and low-grade cytological abnormalities are experiencing CIN2/3 lesion regression remains to be further investigated.

In this cohort, triaging women with ASC-US using HPV testing was ineffective in reducing unnecessary referrals and treatment, primarily due to the high proportion of hrHPV-positive patients (88.8%). This high hrHPV-positive rate can be attributed to several factors. Firstly, the HPV vaccination rate in China remains low [45], resulting in a higher positive rate (about 50-70%) [46–48] of hrHPV among ASC-US women compared to Western countries (about 30-40%) [49–51]. Secondly, as this real-world study involved opportunistic screening in a clinical setting, where patients underwent both HPV and cytology tests, it primarily included high-risk cases. Some hrHPV-negative patients may have been excluded because they did not undergo cytology examination in the study. Thus, the efficacy of HPV testing for triaging ASC-US may be underestimated. However, CISCER assay was negative in all hrHPV-negative patients, and can effectively reduce the referral rate for hrHPV-positive women by 79.5%. This does not change the fact that CISCER assay can complement HPV testing by preventing unnecessary invasive procedures, reduce costs and stress for women, and helping to preserve fertility.

HPV16/18 infections are strongly associated with a higher risk of developing precancerous lesions [52]. However, in this study, 68% (34/50) of HPV16/18-positive women with ASC-US had CIN1- lesions. The specificity of CISCER assay in identifying CIN1- lesions in this population was 94.1%, which can effectively alleviate anxiety in women who have low-grade cytological abnormalities but test positive for HPV16/18. In addition, molecular triage based on CISCER assay was more effective in the non-HPV16/18 group than in the HPV16/18 group. As the number of women receiving the HPV vaccine increases globally, there may be an observation of non-vaccine HPV types progressing to cervical cancer, particularly adenocarcinoma. The results of this study may provide a foundation for future research in the vaccine era. Due to the limited number of hrHPV-negative

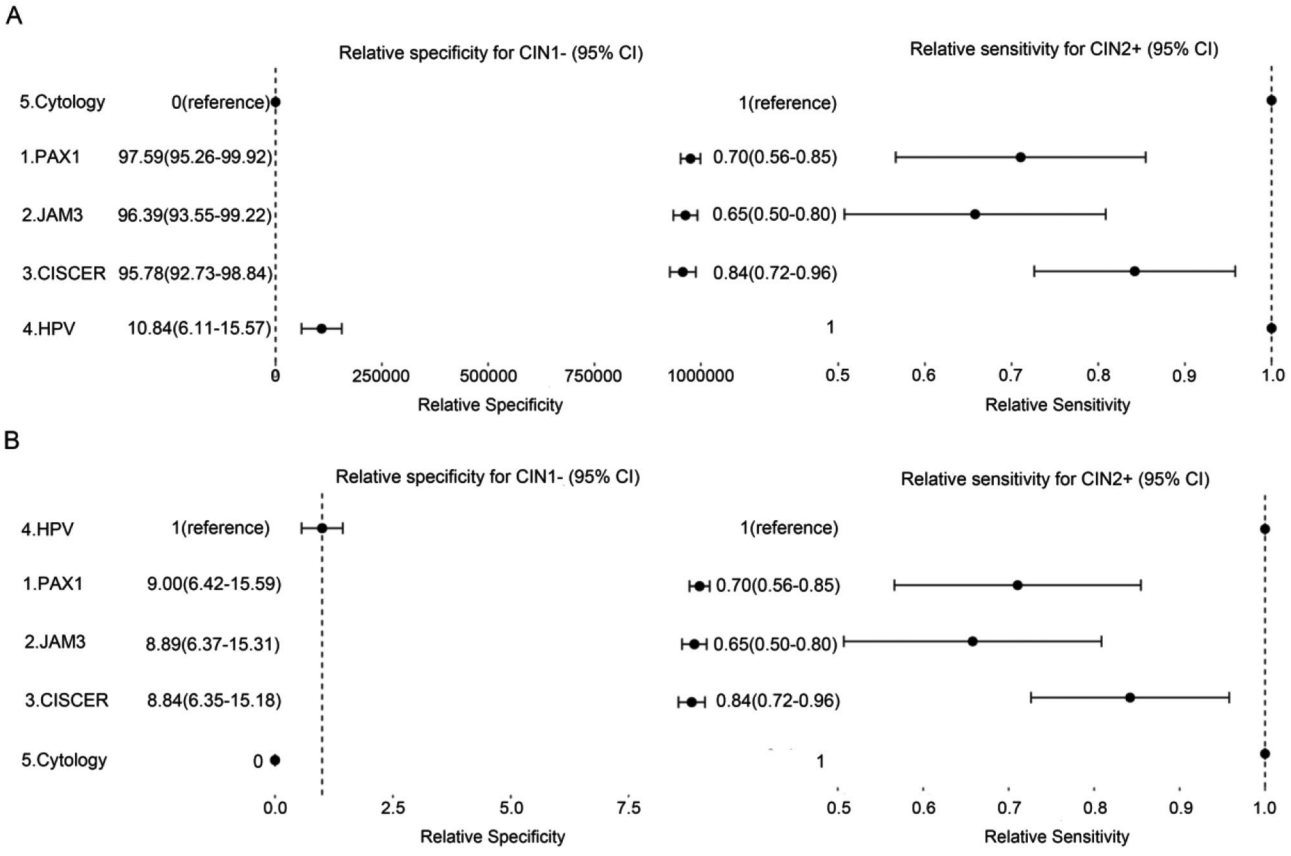

Fig. 4 Forest plots of relative specificity and sensitivity for HPV, cytology and methylation tests in detecting CIN1- or CIN2+lesions. (**A**) Cytology as the reference (**B**) HPV as the reference. Abbreviations: CI, confidence interval; CIN2+: CIN grade 2 or higher; CIN1-: CIN grade 1 or No CIN; HPV: human papillomavirus

patients, high-grade lesions were not observed in this group. While HPV testing is highly sensitive, the possibility of missing high-grade lesions in hrHPV-negative cases [49, 53] should not be overlooked. The methylation test, which is directly correlated with lesion severity, may also help identify high-grade lesions not linked to hrHPV infection.

There are also some limitations in this study: (1) Not all histopathological assessments were based on surgical samples, which may introduce bias; (2) As a real-world and cross-sectional study, not all patients were referred for colposcopy, potentially affecting the assessment of high-grade lesion risk and no follow-up; (3) Subjects were recruited in the colposcopy room and were not women from outpatient clinics; (4) A larger patient sample size and more rigorous clinical trials are needed to evaluate the clinical value of methylation testing.

Conclusions

Our study demonstrates that $PAX1^m$/$JAM3^m$ (CISCER) assay serves as a prominent and reliable supplemental method to HPV-DNA testing for the triage of women with cytologic ASC-US. This assay, characterized by its high sensitivity and specificity, has the potential to serve as a valuable complement to current cervical cancer screening programs. The CISCER assay could significantly reduce unnecessary colposcopy referrals and overtreatment, thereby optimizing patient management and resource utilization in clinical settings. In addition, the molecular triage based on the CISCER assay or single *PAX1* or *JAM3* methylation, had better effects in the women with non-HPV16/18 group. Future research should aim to validate these results in larger and more diverse populations and assess the long-term impacts of this testing approach on the outcomes of cervical cancer screening programs.

Supplementary Information

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Supplementary Material 1

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Author contributions
X.C. and H.J. contributed equally to this paper. H.S. and X.F. proposed and designed this research. X.C. and H.J. collected samples and conducted experiments. H.X. conducted statistical analysis and prepared for the figures and tables. X.C., H.J., L.W., P.L., D.M., and H.W. were responsible for the experimental design and procedures. X.C. and H.J. drafted manuscript. H.S. and X.F. revised manuscript. All authors have read and approved this research to be published.

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Data availability
Data are not publicly available due to its sensitivity (including protected health information of participants). De-identified data may be made available on reasonable request to corresponding author for the purposes of replication of study results.

Declarations

Ethical approval
The name of the Approval Committee or the Internal Review Board (IRB). This research has been approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Human Ethics and Consent to participate declarations
All participants have provided written informed consents according to the Declaration of Helsinki, and which have been approved by the Ethics Committee of Zhejiang Provincial People's Hospital (No.2021SJ020).

Consent for publication
Not applicable.

Clinical trial number
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Competing interests
The authors declare no competing interests.

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