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PAX1/JAM3 methylation in cervical exfoliated cells: a robust diagnostic biomarker for cervical high-grade lesions associated with vaginal dysbiosis

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Abstract

Objective This prospective single-center study aimed to investigate the clinical correlation between *PAX1/JAM3* methylation (CISCER) assay and clinically evaluated vaginal microecological features in women referred for colposcopy, and to assess the potential complementary diagnostic value for detecting high-grade cervical lesions.

Methods Participants were recruited from the Gynecologic Colposcopy Outpatient Clinic of Beijing Anzhen Hospital, Capital Medical University, from February 2024 to December 2024. The clinical performance of the CISCER assay and the vaginal microecology composition of the colposcopy-referred subjects were analyzed.

Results The methylation levels of *PAX1* and *JAM3* were elevated in participants with cervical intraepithelial neoplasia 2 (CIN2). When ΔCt*PAX1* ≤ 6.6 or ΔCt*JAM3* ≤ 10.0, the methylation levels for *PAX1* or *JAM3* were high (with low ΔCt) in ≧CIN2 compared to ≤CIN1 (ΔCt*PAX1*: 4.25 vs. 9.35; ΔCt*JAM3*: 9.34 vs. 13.2) groups, reflecting higher methylation in high-grade lesions. For the detection of CIN2 + and CIN3 + lesions, the CISCER assay showed high discriminative accuracy, with AUC values of 0.826 and 0.852, respectively. Notably, the CISCER assay demonstrated superior specificity compared to hrHPV testing (89.1% vs. 7.3% for CIN2-), addressing a major limitation of current screening. Elevated *PAX1/JAM3* methylation levels are associated with vaginal dysbiosis—characterized by *Lactobacillus* depletion and *Gardnerella* enrichment—and are potential strong predictors of CIN2+.

Conclusions The *PAX1/JAM3* methylation (CISCER) assay for cervical exfoliated cells has robust diagnostic association with high-grade cervical lesions (CIN2+) and correlates with vaginal dysbiosis biomarkers, including *Gardnerella* enrichment and *Lactobacillus* depletion, supporting its potential as a molecular triage tool for stratifying hrHPV-positive women.

Keywords Junctional adhesion molecule (*JAM3*), Paired box gene1 (*PAX1*), Methylation, Cervicovaginal microecology, Human papillomavirus (HPV)

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Background

Cervical cancer (CC) remains a significant public health challenge and is one of the most prevalent gynecological malignancies worldwide, posing a significant burden of incidence and mortality among women worldwide [1]. In China, CC, the predominant reproductive tract malignancy among women, accounted for approximately 150,700 new cases and 55,700 deaths in 2022, a burden compounded by an alarmingly rising incidence in younger age groups [2]. Given the overlap in symptoms like bleeding and discharge between CC, endometrial cancer, and benign diseases, a thorough diagnostic workup should encompass both cervical screening (cytology/HPV testing) and, if indicated, subsequent endometrial imaging (e.g., transvaginal ultrasound), particularly when cervical screening is negative [3–5]. Furthermore, the pathogenesis of CC involves a multistage process driven primarily by persistent infection with high-risk human papillomavirus (hrHPV), particularly HPV16/18, disrupting tumor suppressor pathways and causing epigenetic dysregulation. This synergistically drives the progression from cervical intraepithelial neoplasia (CIN) to invasive carcinoma [6, 7].

Carcinogenesis typically evolves through a prolonged precancerous stage, from low-grade to high-grade CIN, offering a crucial window for intervention through strategies [8, 9]. According to Chinese guidelines for CC screening, primary screening modalities include hrHPV testing, cytology, or a co-testing strategy [10]. However, these modalities face limitations, including suboptimal accuracy in identifying high-grade CIN and more severe (CIN2+), substantial upfront costs, and dependence on specialized cytotechnologists, which hampers scalable implementation in resource-limited settings [10, 11]. Consequently, there is an urgent need for novel technologies and strategies to improve risk stratification among screening positive (e.g., hrHPV) individuals, thereby enabling the precise detection of CIN2+ [12].

Cervical carcinogenesis is driven by interactions between host factors (including vaginal microbiome dysbiosis and epigenetic modifications) and viral determinants [7, 8, 13–17]. Aberrant DNA methylation, particularly promoter CpG island hypermethylation, disrupts transcriptional regulation, driving carcinogenesis [18]. Both host and viral DNA methylation have been reported to play crucial roles in determining the persistence of hrHPV infection [13, 19, 20]. Therefore, DNA methylation biomarkers have attracted attention due to their critical role in diagnosis, evaluation, and treatment strategies [21]. This approach effectively minimizes overtreatment and reduces the risk of missed diagnoses, serving as both a triage tool for CC screening and a management strategy for patients with CIN2+ [8, 22]. Junctional adhesion molecule 3 (*JAM3*) methylation has been implicated in the progression of multiple malignancies and serves as a viable biomarker for detecting CIN2+ [23, 24]. Additionally, the combined methylation analysis of *JAM3* and paired box gene 1 (*PAX1*) has been linked to persistent hrHPV infections (≥ 3 years) and has demonstrated superior triage performance compared to cytology in cervical exfoliated cells from hrHPV-positive patients [25, 26]. Notably, *PAX1/JAM3* methylation ($PAX1^m/JAM3^m$; CISCER) assay also exhibited superior triage ability to CC screening strategies in the women with non-16/18 hrHPV or HPV16/18 groups [26–28]. Interestingly, $PAX1^m/JAM3^m$ assay can effectively detect CIN3 + in younger women with only mildly abnormal cytology, demonstrating high specificity [28].

Studies have indicated that vaginal microbiota richness and diversity relative to HPV infection, CIN severity, while vaginal dysbiosis, characterized by a depletion of *Lactobacillus* species and an overgrowth of pathogens such as *Gardnerella*, disrupts the clearance of HPV through mechanisms involving inflammatory signalling and modulation of oxidative stress [29–31]. *Lactobacilli*, which maintain a mildly acidic vaginal environment by secreting lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins, are crucial for vaginal microecology stability. Notably, an elevated vaginal pH is reported strongly correlated with cervical lesions [32–34]. Bacterial vaginosis has been reported to be associated with higher HPV prevalence, increased disease progression, and delayed viral clearance [35]. Therefore, maintaining vaginal microecology balance is of paramount importance in cervical cancer prevention.

This study leveraged real-world clinical data to evaluate the value of $PAX1^m/JAM3^m$ assay in detecting high grade cervical lesions. Additionally, this study also investigated the association between the vaginal flora composition and the prevalence of HPV infection as well as cervical lesions. The primary objective of this study is to investigate HPV testing, methylation testing, and the vaginal microecology in women with cervical lesions in Beijing city. We aimed to establish a comprehensive clinical database that enhances the detection efficiency of cervical lesions, reduces over-treatment, and alleviates anxiety among HPV-positive women. Furthermore, this clinical research seeks to provide a novel perspective for the precise clinical diagnosis of CC and cervical high-grade lesions.

Materials and methods

Study subjects

This study was a single-center, prospective cross-sectional observational study conducted at the gynecology department of Beijing Anzhen Hospital, Capital Medical University. The study was approved by the Ethics Committee of Beijing Anzhen Hospital, Capital Medical

University (Approval No. KS2023085). Information, including medical data, hrHPV and cytology results, and vaginal microecological features assessed by Gram-stain microscopy, were collected from consecutive women referred for colposcopy due to positive hrHPV, abnormal cytology, or suspicious clinical symptoms, including persistent abnormal vaginal bleeding, postcoital bleeding, unexplained atypical vaginal discharge, or visible abnormalities between February and December 2024. Information on other potential factors such as smoking, sexual behavior, parity, contraceptive use, and prior CIN history was not systematically recorded in the hospital records for this cohort.

This cohort represents a selected referral group enriched for cervical abnormalities rather than a general screening population. Inclusion criteria were: (1) Age ≥ 18 years with a history of sexual activity and no contraindications for cervical sample collection; (2) Signed informed consent form; (3) Completion of colposcopy examination and cervical biopsy with a definitive pathological diagnosis; (4) Women meet any of the following criteria for colposcopy: (a) positive hrHPV result; (b) cytology ≥ ASC-US; (c) abnormal cervical appearance observed during the gynecological exam; or (d) clinical symptoms that the physician deemed necessary for further investigation; (5) No vaginal douching, medication use, or sexual activity within three days prior to examination; (6) Availability of complete clinical records. Exclusion criteria: (1) Menstrual period, pregnancy, or lactation; (2) History of total hysterectomy; (3) Diagnosis and treatment of reproductive tract tumors, other malignancies or concurrent multiple cancers; (4) Severe systemic or immune disorders; (5) Vaginal douching or medication usage within one week.

Colposcopy examination and pathology

The participant was positioned in the lithotomy position. A sterile disposable speculum is inserted into the vagina and used to fully expose the cervix, vaginal walls, and fornices, allowing for visualization of the cervix. The cervix was treated with 3% to 5% acetic acid and Lugol's iodine solution to evaluate acetowhite and iodine staining patterns under colposcopic magnification. Subsequently, 2 to 4 targeted biopsies were obtained from areas displaying the most suspicious changes. Additionally, endocervical curettage (ECC) was performed in cases where transformation zone (TZ) II and III was identified or if there was any uncertainty. In other circumstances, 1 to 2 random biopsies were performed with the consent of the participants.

Specimens collected from different sites were accurately labeled and fixed in 4% neutral buffered formaldehyde. The histological diagnosis of CIN was established based on morphological features observed in hematoxylin and eosin-stained sections, following WHO Classification of Tumours of the Female Reproductive Organs (4th edition, 2014). All tissue pathology slides were independently reviewed by two experienced pathologists who were blinded to methylation and microbiology results. Pathological diagnoses from different biopsy sites were reported separately, with each site specified individually, particularly when lesion grade differed. The histopathological staging was classified into the following categories: Normal (women with normal pathology referred for colposcopy), CIN1, CIN2, CIN3, squamous cell carcinoma (SCC), and adenocarcinoma (AD). Management of cervical precancerous lesions or cervical cancer adhered to the Chinese standard guidelines [36–38].

Cytology tests

The exfoliated cell samples were collected using CervexBrush (Rovers Medical Devices, Oss, the Netherlands), which was inserted into the cervix and rotated clockwise 5–6 times. The samples were then preserved in PreservCyt solution (Hologic, Bedford, MA, USA) for cytology examination. Cytology examinations were performed by qualified cytopathologists and classified according to the Bethesda System (third edition, 2014) as follows: negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), atypical squamous cells—cannot exclude HSIL (ASC-H), atypical glandular cells (AGC), adenocarcinoma in situ (AIS), squamous cell carcinoma (SCC), and adenocarcinoma (AD).

High-risk HPV testing

The residual exfoliated cell samples from the preservation solution were detected using hrHPV Genotyping Real Time PCR Kit (Shanghai ZJ Bio-Tech Co., Ltd., Shanghai, China) following the manufacturer's instructions. The assay identifies HPV 16 and HPV 18 individually, and additional genotypes (including 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Vaginal microecology analysis

After fully exposing the cervix, vaginal walls, and fornices with a sterile disposable speculum, vaginal secretions were collected from the vaginal wall using a sterile swab, placed in a sterile test tube, and labeled for laboratory analysis. Samples were Gram-stained (HealthBless Bio-Tec. Co., Ltd., Shenzhen, China) and examined microscopically. Gram-staining provides only semi-quantitative data and lacks the species-level resolution offered by 16 S rRNA gene sequencing. Morphological and functional assessments were performed based on the "Expert Consensus on the Clinical Application of Vaginal

Microecology Testing" [39], including bacterial density, grade, dominant species, pathogenic microorganisms, and disease-related scoring. Functional indicators, such as metabolic byproducts and enzymatic activity, were also evaluated.

Normal vaginal microecology parameters included: *Lactobacillus* as the predominant species, bacterial density (II, III), diversity (II), *Lactobacillus* abundance (++/+++), Nugent score (0–3), leukocyte count (< 5 per high-power field), vaginal pH of 3.8–4.5, aerobic vaginitis (AV) score (0–2), and negative results for leukocyte esterase, sialidase, polyamines, and hydrogen peroxide. Dysbiosis was defined by any deviation from these parameters. Two research assistants independently recorded and verified all data. Additionally, routine gynecological microbiological assessments were conducted in 172 patients through routine vaginal leucorrhea examination and microecological assessment prior to their colposcopy examination. Density and diversity were graded on Gram-stained vaginal smears according to established China expert consensus [39]. Briefly, density (reflecting the total bacterial biomass) was graded based on the average number of bacteria per high-power field under the oil-immersion lens (10 × 100 magnification): Grade I (1–9), Grade II (10–99), Grade III (≥ 100/full field), and Grade IV (dense aggregates or confluent coverage of epithelial cells). Diversity was graded based on the number of distinct bacterial morphotypes: Grade I (1–3 types), Grade II (4–6), Grade III (7–9), and Grade IV (≥ 10). Thus, density is defined as an estimate of bacterial load per field rather than an absolute molecular quantification.

Microecology assessment was performed only when clinically indicated and when patients consented to the test; specifically, clinicians ordered microbiological testing (Gram-stain microscopy) based on clinical judgment: patients had symptoms or signs suggestive of vaginitis. Therefore, microbiological data were available for a subset of 172 participants who underwent this specific assessment based on clinical indications or physician orders.

Sample collection and *PAX1*/*JAM3* methylation (CISCER) assay

In the colposcopy outpatient clinic, gynecologists performed the sample collected for methylation assay. A Cervical Sampling Brush (HUALAISI Medical Devices, Ningbo, China), which was rotated clockwise 5 to 6 times within the cervix and endocervical canal. Exfoliated cells were immediately preserved in CidaBio Cell Preservation solution (Cidabio Biotech., Guangzhou, China) at the time of collection and stored at 4 °C for short-term transport prior to DNA extraction. All laboratory personnel were blinded to any information of regarding the clinical diagnosis.

Genomic DNA (gDNA) was extracted from cervical exfoliated cell samples preserved in the preservation solution using the JH-DNA Isolation and Purification Kit (OriginPoly Bio-Tec Co., Ltd., Beijing, China), following the manufacturer's instructions. DNA extraction, bisulfite conversion, and methylation analysis of *PAX1* and *JAM3* were followed manufacturer protocols with internal positive and negative controls, as described previously [12, 26]. Briefly, the hyper-methylation levels of *PAX1* or *JAM3* were detected using the Human *PAX1* and *JAM3* Dual-Gene Methylation Detection Kit (OriginPoly Bio-Tec Co., Ltd., Beijing, China; National Medical Products Administration (NMPA) of China Class III medical device No. 20233400253), with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal reference. The hypermethylation levels of *PAX1* and *JAM3* were determined by the difference in cycle threshold values (ΔCt), calculated as $\Delta Ct_{PAX1} = Ct_{PAX1} - Ct_{GAPDH}$ and $\Delta Ct_{JAM3} = Ct_{JAM3} - Ct_{GAPDH}$. In this study, the positivity of CISCER (commercial name of PAX1/JAM3 dual-gene methylation kit) was defined as $\Delta Ct_{PAX1} \leq 6.6$ or $\Delta Ct_{JAM3} \leq 10.0$ (i.e., a sample was considered positive if either marker met the predefined threshold). CISCER negative criteria was $\Delta Ct_{PAX1} > 6.6$ and $\Delta Ct_{JAM3} > 10.0$. The clinical assay procedures for *PAX1* and *JAM3* methylation including all laboratory quality control steps, were carried out according to expert guidelines [40]. To avoid data-driven overfitting, the positivity cut-offs (ΔCtPAX1 ≤ 6.6, ΔCtJAM3 ≤ 10.0) were pre-defined by the assay manufacturer, approved by NMPA of China, and validated in prior clinical trial [12, 26, 41], and were not derived or optimized based on the dataset from the current study. However, optimal thresholds may vary across diverse racial populations. Accordingly, in accordance with medical device regulations across different countries, we recommend external validation of these cut-offs in distinct population groups prior to broad clinical implementation. A lower Ct (and therefore lower ΔCt) indicates a higher starting concentration of the target sequence, corresponding to greater methylation of the target gene.

Statistical methods

All statistical analyses were performed using R software (version 4.4.0). The Receiver Operating Characteristic (ROC) curve construction and calculation of the receiver operating characteristic curve (AUC) values with 95% Confidence Intervals (95% CI) were implemented through the pROC package (version 1.18.4). Diagnostic performance metrics including sensitivity and specificity, along with their 95% CI, were derived using functions from the epiR package (version 2.0.75). Pairwise comparisons

of correlated AUCs were performed using DeLong's test where indicated. Categorical data were expressed as frequency percentages, and group comparisons were analyzed using Chi-square tests or Fisher's exact probability tests as appropriate. We assessed distributional assumptions visually (histograms and QQ plots) and using the Shapiro-Wilk test where appropriate. As the Ct values and bacterial scores did not follow a normal distribution, non-parametric tests, Kruskal-Wallis, were employed for between-group comparisons and were summarized using median values with interquartile ranges (Q1-Q3). Analyses were performed as complete-case analyses including samples that passed laboratory QC and had histopathology available. Correlations between ΔCt values, histopathology grade and microecological parameters were assessed using Spearman's rank correlation coefficient. To account for multiple hypothesis testing in the correlation/heatmap analyses, P values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method. Statistical significance was determined P value < 0.05.

Results

This study included 300 women who underwent colposcopy due to abnormal screening results or clinical symptoms. $PAX1^m$/$JAM3^m$ assay was performed on cervical exfoliated cell samples from 294 patients with signed informed consent. Finally, a total 264 women were included in the data analysis (Fig. 1, Table 1). The baseline characteristics of the 172 participants who underwent clinician-directed vaginal microecological evaluation in Supplementary Table S4. There were no significant differences in age or TZ observed among women with pathological normal/inflammatory/CIN1 (CIN1-) and CIN2 + groups. The median age of the women in this cohort was 42 years (range: 33–54), with a predominant proportion classified as TZ III (66.7%). Pathological analysis of the 71 cases for CIN2 + revealed CIN2 (42.3%), CIN3 (49.3%), SCC (7.0%), and AD (1.4%). Among women with CIN2+, 93% tested positive for hrHPV, with 50.7% ($n = 36$) for HPV16/18 positive —a significant increase compared to 33.7% in the CIN1- group ($p < 0.05$). Cytology demonstrated 31% NILM and 18.3% ASC-US in CIN2 + group. The ΔCt values of the *PAX1* or *JAM3* decrease as the severity of lesion grades increases in Fig. 2. In addition, the methylation levels for *PAX1* or *JAM3* were high (with low ΔCt) in CIN2 + compared to CIN1- (Δ*PAX1*: 4.25 vs. 9.35; Δ*JAM3*: 9.34 vs. 13.2) groups, reflecting higher methylation level in high-grade lesions. In Table S1, the hrHPV testing showed superior sensitivity, achieving rates of 93% for CIN2 + and 95.1% for CIN3+. However, it exhibits the lowest specificity compared to other tests. Additionally, a negative hrHPV result was observed in the AD case. The sensitivity of $PAX1^m$ or $JAM3^m$ tests were 66.2% (95% confidence interval: 55.2–77.2%) or 57.7% (46.3–69.2%) for women with CIN2+, respectively. The CISCER assay achieved the highest AUC at 0.826 (0.754 – 0.897) and negative predictive value (NPV) at 91% (86.9–95.1%) for CIN2+. The sensitivity of CISCER assay reached 76.1% (66.1–86%) and reasonable specificity at 89.1% (84.7–93.5) in CIN2+,

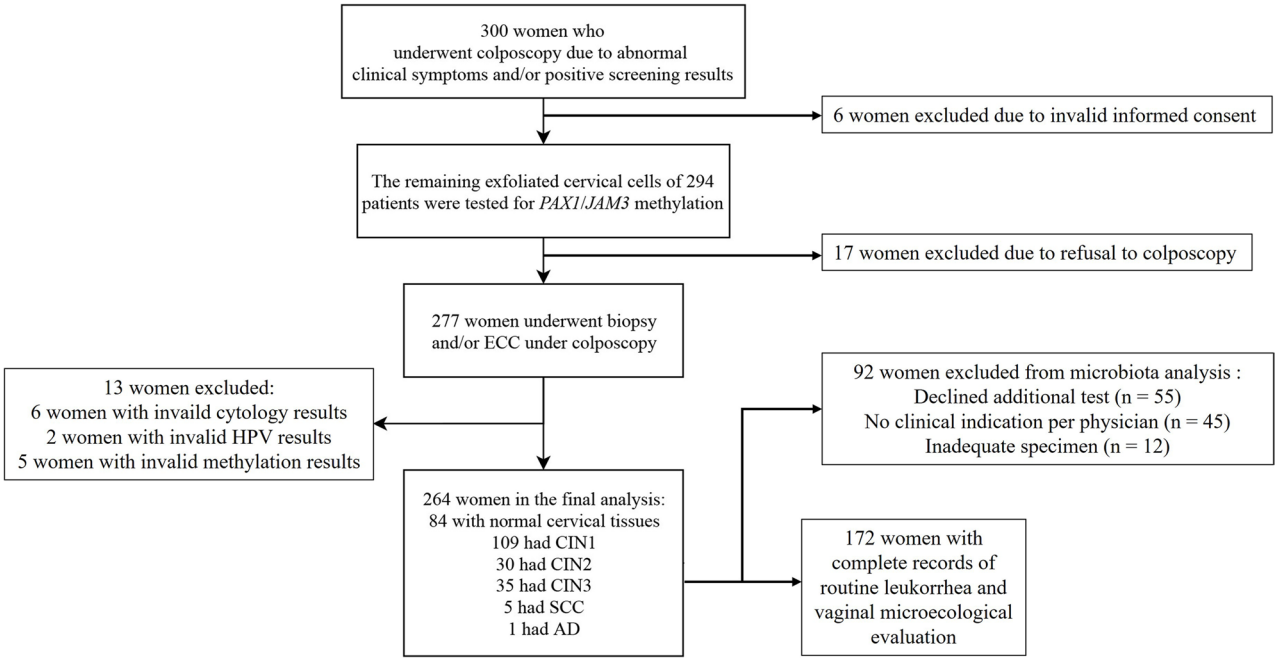

Fig. 1 Flowchart of the study. Abbreviations: CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; AD: adenocarcinoma; JAM3: junctional adhesion molecule 3; PAX1: paired box gene 1

Table 1 Clinical characteristics of the participants

	Overall (*N* = 264)	CIN1- (*N* = 193)	CIN2+ (*N* = 71)	*P* value
Age				0.9
Median [Q1,Q3]	42.0 [33.0,54.0]	41.7 [32.7,54.0]	42.9 [34.8,52.5]	
TZ				0.8
I	43 (16.3%)	30 (15.5%)	13 (18.3%)	
II	45 (17.0%)	34 (17.6%)	11 (15.5%)	
III	176 (66.7%)	129 (66.8%)	47 (66.2%)	
Pathology				< 0.001
Normal	84 (31.8%)	84 (43.5%)	0 (0%)	
CIN1	109 (41.3%)	109 (56.5%)	0 (0%)	
CIN2	30 (11.4%)	0 (0%)	30 (42.3%)	
CIN3	35 (13.3%)	0 (0%)	35 (49.3%)	
SCC	5 (1.9%)	0 (0%)	5 (7.0%)	
AD	1 (0.4%)	0 (0%)	1 (1.4%)	
hrHPV				0.036
Negative	19 (7.2%)	14 (7.3%)	5 (7.0%)	
HPV 16/18	101 (38.3%)	65 (33.7%)	36 (50.7%)	
Non-16/18	144 (54.5%)	114 (59.1%)	30 (42.3%)	
hrHPV				
LBC				< 0.001
NILM	123 (46.6%)	101 (52.3%)	22 (31.0%)	
ASC-US	61 (23.1%)	48 (24.9%)	13 (18.3%)	
AGC	1 (0.4%)	1 (0.5%)	0 (0%)	
LSIL	43 (16.3%)	33 (17.1%)	10 (14.1%)	
ASC-H	13 (4.9%)	6 (3.1%)	7 (9.9%)	
HSIL	22 (8.3%)	3 (1.6%)	19 (26.8%)	
AD	1 (0.4%)	1 (0.5%)	0 (0%)	
ΔCt *PAX1*				< 0.001
Median [Q1,Q3]	8.40 [6.27,11.4]	9.35 [7.32,12.6]	4.25 [1.36,7.87]	
ΔCt *JAM3*				< 0.001
Median [Q1,Q3]	12.7 [10.8,14.1]	13.2 [11.6,14.4]	9.34 [4.34,12.5]	
CISCER				< 0.001
Negative	189 (71.6%)	172 (89.1%)	17 (23.9%)	
Positive	75 (28.4%)	21 (10.9%)	54 (76.1%)	

Non-16/18 hrHPV genotypes: HPV31/33/35/39/45/51/52/56/58/59/66/68; CISCER either positive criteria: ΔCt*PAX1* ≤ 6.6 or ΔCt*JAM3* ≤ 10.0; CISCER negative criteria: ΔCt*PAX1* > 6.6 and ΔCt*JAM3* > 10.0; CIN1-: normal and CIN1; CIN2+: CIN2, CIN3, in-situ carcinoma, and cancer

Abbreviations: *TZ* Transformation zone, *CIN* Cervical intraepithelial neoplasia, *SCC* Squamous cell carcinoma, *AD* Adenocarcinoma, *LBC* Liquid-based cytology, *NILM* Negative for intraepithelial lesion or malignancy, *ASC-US* Atypical squamous cells of undetermined significance, *AGC* Atypical glandular cells, *LSIL* Low-grade squamous intraepithelial lesion, *ASC-H* Atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion, *HSIL* High-grade squamous intraepithelial lesion, *HPV* Human papillomavirus, *hrHPV* High-risk HPV

surpassing that of cytology (69% at ≥ ASC – US, 50.7% at ≥ LSIL, and 26.8% at ≥ HSIL) and HPV16/18(+) at 50.7%, respectively. Moreover, CISCER assay exhibited 87.8% (77.8 – 97.8%) sensitivity and good specificity at 82.5% (77.5–87.5%) in CIN3+, with all positive results observed in both SCC and AD (Fig. 3, TableSI). In contrast, cytological analysis of these cancer cases revealed two NILM and two ASC-US results. Table S5 presented the characteristics of the 84 women with normal histology stratified by hrHPV status (HPV-negative: $n = 6$; HPV16/18: $n = 32$; non-16/18 hrHPV: $n = 46$). Although the majority of this normal group were hrHPV-positive (78/84, 92.9%), only a small proportion had positive methylation results (PAX1 methylation: 5/84, 6.0%; CISCER positive: 6/84, 7.1%).

To further explore the microecological characteristics of participants with high-grade cervical lesions or cancer, the routine leukorrhea and vaginal microecological data of 172 women were analyzed (Table 2 and Table S2). The cervical lesions group exhibited a higher proportion of abnormal leucorrhea in color and quality compared to the normal group, although this did not reach a statistically significant difference. The proportion of abnormal vaginal cleanliness did not increase in patients with CIN2+, but there was a significant rise in leukocyte count. The pH of CIN3 + patients was significantly higher than that of the other groups. Notably, *Lactobacillus* was much lower, while *Gardnerella* was very common in patients with cervical high-grade lesions. Other bacteria like clue cells, *Candida*, *Actinomyces*, *Enterobacter*, *Streptococcus*, and other gram-positive bacteria were rarely seen. *Trichomonas* vaginalis and *Staphylococcus* were not found in the study. As a result, the Nugent score, AV status, and vaginal flora composition and microbial community structure differed between the Normal group and the various lesion groups.

A correlation analysis was performed between the patients' cervical screening results and vaginal microecological outcomes (Fig. 4), with the scoring method as shown in Table S3. The results revealed that the pathological outcomes of high-grade cervical lesions were negatively correlated with ΔCt*PAX1* ($r = -0.552$, $P < 0.001$), ΔCt*JAM3* ($r = -0.607$, $P < 0.001$), *Lactobacillus* ($r = -0.316$, $P < 0.001$), and microecological grade ($r = -0.111$, $P < 0.001$). It was positively correlated with LBC ($r = 0.370$, $P < 0.001$), leukocyte ($r = 0.251$, $P < 0.001$), *Gardnerell*a ($r = 0.354$, $P < 0.001$), Nugent score ($r = 0.499$, $P < 0.001$), AV ($r = 0.213$, $P = 0.004$), pH ($r = 0.283$, $P < 0.001$), polyamine ($r = 0.251$, $P < 0.001$), sialidase ($r = 0.321$, $P < 0.001$), and H_2O_2 ($r = 0.339$, $P < 0.001$). In addition, the methylation levels were significantly associated with microbiome dysbiosis.

Discussion

Epigenetic alterations, particularly the hypermethylation of specific genes, have gained prominence as molecular biomarkers for cervical carcinogenesis [42]. Concurrently, perturbations or even disruptions in vaginal microecological composition and balance have been implicated in cervical disease progression and carcinogenesis [43]. While extensive investigations delineated the independent roles of DNA methylation patterns and

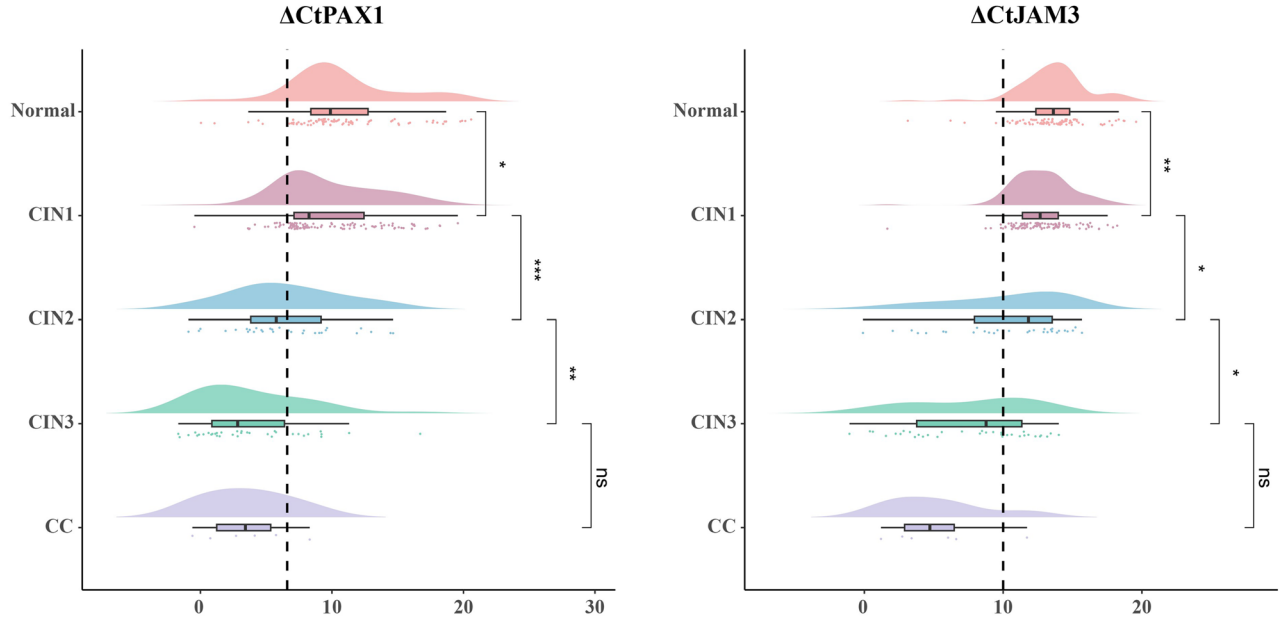

Fig. 2 Distribution of ΔCt values for methylation of *PAX1* and *JAM3* in different pathological groups ΔCt = Ct_target − Ct_GAPDH; lower ΔCt indicates higher methylation. Dashed lines indicate predefined positivity cutoffs (ΔCtPAX1 ≤ 6.6; ΔCtJAM3 ≤ 10.0). Sample sizes = 264; Statistical tests: Kruskal-Wallis test; Abbreviations: CIN: cervical intraepithelial neoplasia; CC: cervical cancer; JAM3: junctional adhesion molecule 3; PAX1: paired box gene 1. Statistical tests: Kruskal-Wallis test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns $P > 0.05$

microbial dysbiosis in cervical pathology, integrative analyses elucidating their associations remain scarce. Our study employed *PAX1*/*JAM3* methylation profiling in the colposcopy-referred cohort, demonstrating superior discriminatory capacity in differentiating normal/low-grade lesions (CIN1-) from high-grade lesions and carcinoma (CIN2+), showing a higher level of specificity than HPV testing. Additionally, this is the first study to report that methylation levels clearly association with dysbiosis of the vaginal flora (the factors marked by high pH, *Gardnerella* enrichment, and *Lactobacillus* depletion), suggesting a co-occurring disruption of the local microenvironment during the lesion progression. This specific dysbiosis of the vaginal flora was observed in high-grade lesions, further highlighting the interconnected landscape of epigenetic alteration and vaginal microenvironment imbalance in cervical carcinogenesis.

In a healthy vaginal microbiome, *Lactobacillus* species dominated, exerting protective effects by producing lactic acid, hydrogen peroxide, and bacteriocins, which prevented pathogen adhesion and suppressed pathogenic bacteria overgrowth [44, 45]. Literature reported that in the state of HPV infection and cervical lesions, the cervical-vaginal microecological becomes imbalanced. This imbalance is characterized by an increase in pH levels, alterations in microbial diversity, and a substantially enhancement of pathogenic anaerobic bacteria, including *Gardnerella*, *Atopobium*, *Prevotella*, and *Sneathia* [35, 46]. Microbiota dysbiosis exhibited intricate associations with oncogenic pathways, particularly in the context of hrHPV infection and cervical carcinogenesis [29, 47]. In our study, *Gardnerella* levels increased with cervical lesion severity, alongside reduced other microbial abundance and strongly *Lactobacillus* depletion. The literature evidence indicates that vaginal dysbiosis increases HPV susceptibility by promoting viral colonization, persistent infection, and tumor progression. Bidirectional interactions emerged as HPV infection perpetuates microbial imbalance [48, 49]. A dysregulated microbiome contributed to chronic inflammation, which upregulated HPV oncoprotein expression, thereby accelerating cellular transformation and genomic instability. Moreover, oxidative stress and reactive oxygen species (ROS) accumulation could directly damage DNA, impair repair mechanisms, and facilitate HPV DNA integration into the host genome [50–52]. However, the limited number of HPV-negative cases in this study precluded establishing a statistically correlation between HPV status and vaginal microecological composition.

Beyond diagnostics, the *Lactobacillus* depletion observed in our study highlights a potential therapeutic target. Probiotic supplementation, particularly with specific *Lactobacillus* strains, may help restore a healthy vaginal microenvironment and promote HPV clearance by modulating local immunity and epithelial integrity [53–56]. Therefore, correcting microecological dysbiosis represents a complementary avenue worthy of future investigation alongside molecular triage tools like the methylation assay.

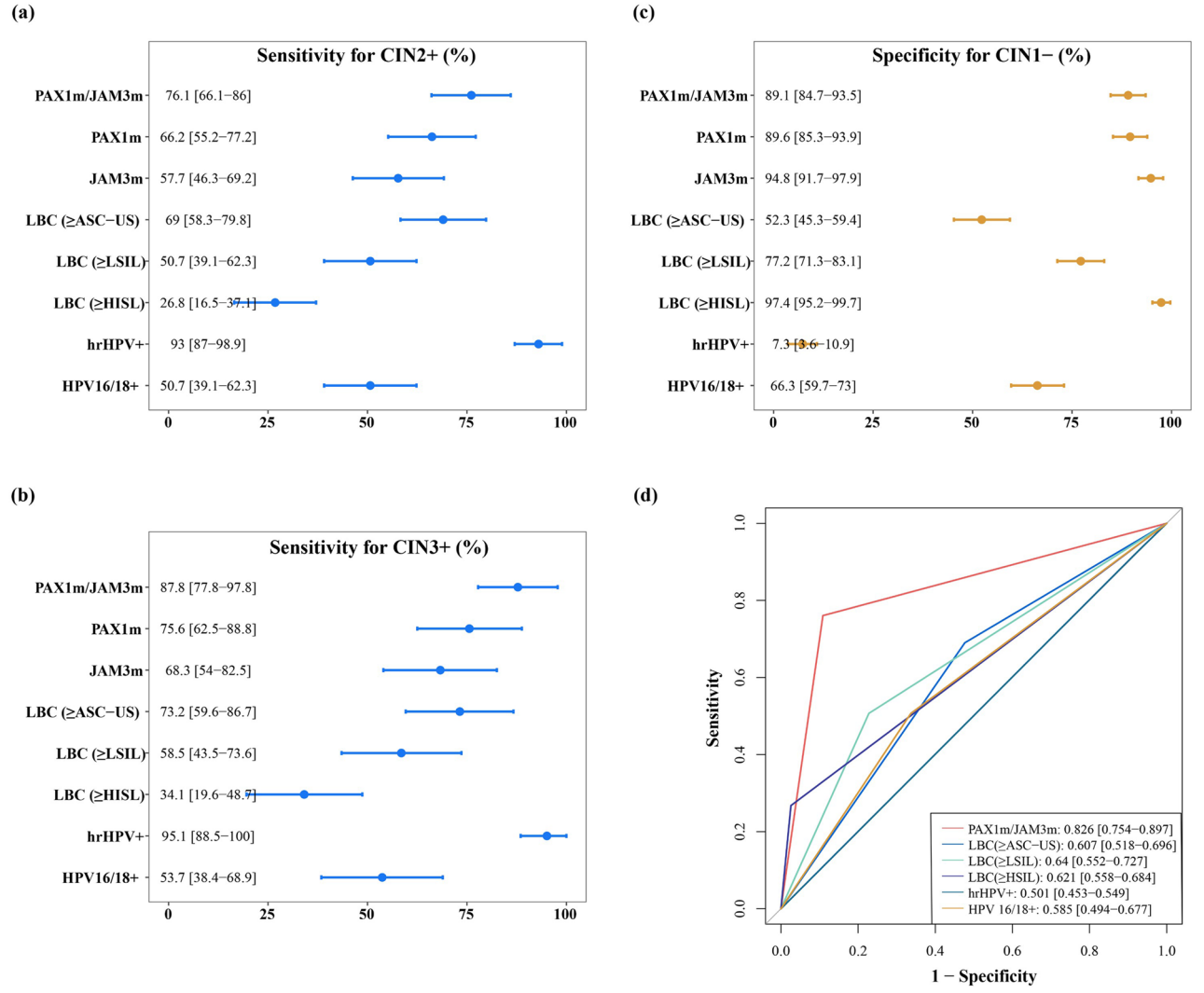

Fig. 3 Clinical efficacy of different screening methods for triaging cervical lesions (**a**) The sensitivity for CIN2+; (**b**) The sensitivity for CIN3+; (**c**) The specificity for CIN1-; (**d**) ROC curve of each clinical parameter for identifying CIN2+. Abbreviations: CIN: cervical intraepithelial neoplasia; ASC-US: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; LBC ≥ ASC-US: the positive cytology results were included ASC-US, LSIL, ASC-H, HSIL, and CC; LBC ≥ LSIL: the positive cytology results were included LSIL, ASC-H, HSIL, and CC; LBC ≥ HSIL: the positive cytology results were included HSIL, and CC; JAM3: junctional adhesion molecule 3; PAX1: paired box gene 1; PAX1m: the methylation of PAX1 gene; JAM3m: the methylation of JAM3 gene; hrHPV: high-risk human papillomavirus; LBC: liquid-based cytology

Table 2 Results of routine leucorrhea and vaginal microecology in 172 women

Characteristics	Unit	Normal [*N*(%)] *N* = 62	CIN1 [*N*(%)] *N* = 55	CIN2[*N*(%)] *N* = 23	CIN3+[*N*(%)] *N* = 32	*P* value
Abnormal leucorrhea color		11 (18%)	12 (22%)	5 (22%)	13 (41%)	0.093
Abnormal leucorrhea quality		3 (4.8%)	4 (7.3%)	3 (13%)	6 (19%)	0.13
Leukocyte	0–5/HP	60 (97%)	52 (95%)	20 (87%)	25 (78%)	0.014
	5–15/HP	2 (3.2%)	3 (5.5%)	3 (13%)	7 (22%)	
Dominant bacteria	Negative	4 (6.5%)	1 (1.8%)	1 (4.3%)	7 (22%)	< 0.001
	Gardnerella	2 (3.2%)	4 (7.3%)	3 (13%)	11 (34%)	
	Cocci	4 (6.5%)	5 (9.1%)	0 (0%)	3 (9.4%)	
	Lactobacillus	52 (84%)	45 (82%)	19 (83%)	11 (34%)	
pH		4.2 (4.0, 4.2)	4.2 (4.2, 4.4)	4.2 (4.0, 4.5)	4.5 (4.3, 4.8)	< 0.001
H_2O_2	+	11 (18%)	30 (55%)	11 (48%)	21 (66%)	< 0.001

Abbreviations: *N* Number, *pH* hydrogen ion concentration, *HP* High power field

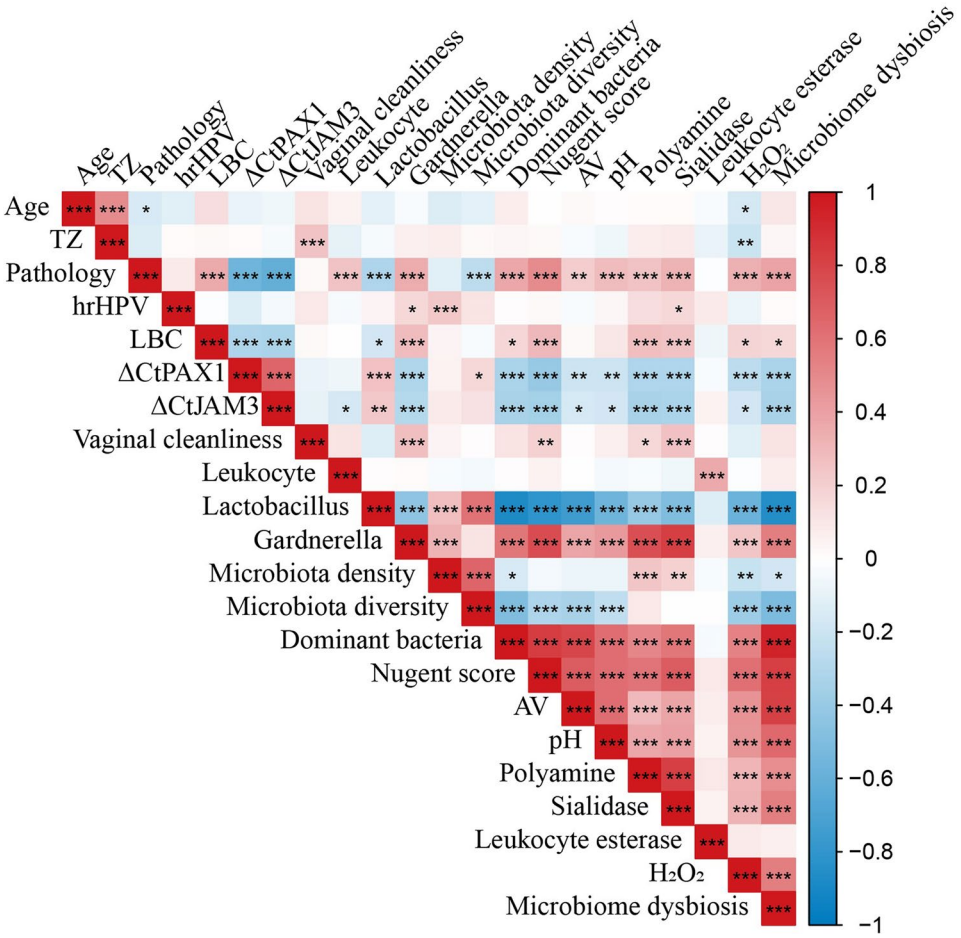

Fig. 4 The correlation heatmap of clinical indexes Tile color encodes Spearman correlation coefficients (*r*) on a continuous scale from + 1 (deep red, strong positive correlation) through 0 (white, no correlation) to − 1 (deep blue, strong negative correlation), and the numeric value in each tile is the corresponding *r*. Statistical significance is indicated by: **P* < 0.05; ***P* < 0.01; ****P* < 0.001 — tiles without asterisks are not statistically significant. Abbreviations: LBC: liquid-based cytology; JAM3: junctional adhesion molecule 3; PAX1: paired box gene 1; TZ: transformation zone; AV: aerobic vaginitis; hrHPV: high-risk human papillomavirus; Sample sizes = 172; Statistical tests: Spearman's rank correlation coefficient

Our study first demonstrated that the progression of cervical lesions is obviously associated with persistent HPV infection, increased DNA methylation, and dysbiosis of the vaginal flora—characterized by *Gardnerella* enrichment and *Lactobacillus* depletion—which co-occur with disease advancement (Fig. 4). Specifically, elevated DNA methylation levels (by a decrease in ΔCt of *PAX1* and *JAM3*) showed an association with microbial dysbiosis, reflecting a potential bidirectional relationship where microbial shifts parallel epigenetic changes that further accelerate carcinogenic pathways, analogous to established mechanisms in colorectal cancer development [57]. This microbial influence on oncogenesis is mediated through epigenetic reprogramming—including DNA methylation and histone modifications—of host epithelial cells, often initiated by specific pathogens such as *Gardnerella vaginalis*, *Megasphaera micronuciformis*, or *Atopobium vaginae*, which are implicated in gynecological carcinogenesis [57]. Certain microbial metabolites might influence genome-wide DNA methylation patterns and gene expression [57, 58]. Conversely, shifts in methylation signatures might also reshape microbial communities, influencing disease susceptibility [59]. Together, these studies suggest that a bidirectional interaction may exist, where microbial shifts and host epigenetic changes mutually reinforce each other. However, it is also possible that both phenomena are parallel consequences of persistent hrHPV infection, as the virus and the ensuing inflammatory response can independently contribute to microbiome alterations and aberrant host DNA methylation [13, 30, 57]. Future specific mechanisms such as whether treating dysbiosis reverses methylation remain to be elucidated this interplay in future studies. But whatever the mechanism between them, vaginal microecological assessment might demonstrate diagnostic auxiliary in resolving discordant results between HPV testing, cytology, and methylation assays.

Microbial metabolites and inflammatory mediators might modulate host epigenetic machinery. For instance, short-chain fatty acids like butyrate inhibit histone deacetylases, while lipopolysaccharide (LPS) from Gram-negative bacteria triggers inflammatory cytokines that alter DNA methyltransferases activity—both promoting aberrant methylation of tumor suppressor genes [60]. Pathogens such as Chlamydia trachomatis and Mycobacterium species can also induce DNA methylation or histone modifications that co-occur with oncogenic pathways [61, 62]. These mechanisms collectively provide a plausible basis for the association we observed between vaginal dysbiosis (*Gardnerella* enrichment/*Lactobacillus* depletion) and increased *PAX1/JAM3* methylation levels. However, it should be noted that in this experiment, microbiological testing was performed in a clinically indicated subset (n = 172). Symptomatic women are likely overrepresented in the clinically indicated microecology subgroup. Consequently, the prevalence of dysbiosis and inflammatory markers in this subset may be higher than asymptomatic or normal women, which could influence the observed associations between dysbiosis and methylation.

Recent evidence underscores that the landscape of CC prevention and early-stage management has evolved substantially. Wide implementation of HPV vaccination and organized screening programs has reduced disease burden, while advances in diagnostic stratification (including HPV testing and targeted colposcopy) permit more individualized treatment decisions [63]. Concurrently, surgical strategies are shifting toward less radical and fertility-preserving approaches for carefully selected low-risk patients, and sentinel lymph-node mapping is increasingly used to minimize morbidity associated with nodal assessment [64]. The increasing complexity of clinical decision-making has intensified the need for highly accurate triage tools that can reliably distinguish between lesions requiring immediate intervention and those suitable for conservative management or watchful waiting. Our findings illustrated that $PAX1^m$/$JAM3^m$ assay is associated with CIN2 + and correlates with markers of vaginal dysbiosis (e.g., *Gardnerella* enrichment and *Lactobacillus* depletion). This vaginal microecological features and epigenome crosstalk manifested in high-grade cervical lesions, underscoring the therapeutic potential of targeted microbial modulation in arresting the oncogenic progression of premalignant lesions.This crosstalk suggests that integrating host-epigenetic biomarkers (e.g., *PAX1*/*JAM3* methylation) with microecological assessment could provide complementary risk-stratification information, enhancing the accuracy of identifying high-risk patients beyond conventional methods like HPV testing or cytology alone. For instance, methylation levels of *PAX1* and *JAM3* increase progressively with lesion severity and are linked to persistent HPV infection and dysbiotic microbial shifts, both of which contribute to carcinogenic pathways. Combining these molecular markers could thus improve triage strategies, reduce unnecessary colposcopy referrals, and support personalized management of cervical precancerous lesions. While these correlations were statistically significant, the magnitude of the correlation coefficients for some parameters (e.g., microecological grade and leukocyte count) was modest (r < 0.3), suggesting that while these factors are related, they likely represent part of a complex multifactorial microenvironment rather than serving as standalone strong predictors.

Importantly, our normal control group (pathology finding) consisted of women who were referred to colposcopy (largely because of abnormal screening results) and therefore over-represents hrHPV-positive individuals. Despite a high prevalence of hrHPV among these referral controls (78/84, 92.9%), *PAX1/JAM3* methylation and the combined CISCER assay were less positive, which supports the high specificity of methylation testing for histologically established high-grade lesions. These findings suggest that the CISCER (*PAX1* and *JAM3* methylation) assay discriminates histologic disease from transient or screening-detected hrHPV infection and is therefore a promising triage tool in referral populations. Because our cohort is colposcopy-referred and enriched for disease, the diagnostic accuracy estimates we report pertain to a high-prevalence referral setting and some test metrics (e.g., specificity and AUC) may not translate directly to primary screening programs. And this associations among epigenetic, microbial alterations and cervical lesions warrants further investigation to delineate its temporal dynamics and causal relationships. Hence, population-based validation studies are needed before broad implementation.

Interestingly, mechanistic studies provide further context for the distinct diagnostic behaviors of *PAX1* and *JAM3* observed in our cohort, in which *JAM3* demonstrated higher specificity but lower sensitivity—consistent with previous reports [26, 41, 65]. *PAX1* had been implicated in tumor suppression through modulation of oncogenic kinase cascades and has been linked with interactions involving DNA methyltransferases, which may underlie early methylation changes in carcinogenesis [66]. In contrast, *JAM3* had been reported to promote CC metastasis via activation of the HIF-1α/VEGFA pathway and to influence epithelial–mesenchymal transition [67]; as a multifunctional adhesion molecule, *JAM3* is also involved in immune-related processes [67]. These biologic differences — PAX1 being more closely tied to early methylation-mediated growth regulatory pathways and JAM3 to later-stage invasive signaling — could plausibly contribute to the pattern we observe clinically.

Taken together, the complementary biology of the two genes supports a combined triage approach in which a sensitive marker (*PAX1*) identifies more cases while a more specific marker (*JAM3*) reduces false positives, thereby improving overall diagnostic discrimination. While the cut-off values used in this study were validated for the Chinese population and approved by NMPA, we recommend that international implementation of this assay should be preceded by population-specific validation to ensure optimal diagnostic performance in diverse ethnic groups.

Based on our findings, we propose a potential triage algorithm: In a potential screening-based workflow, women who test hrHPV-positive could undergo CISCER (*PAX1* and *JAM3* methylation) assay. Patients who are CISCER-positive would be prioritized for immediate colposcopy and further diagnostic workup, whereas CISCER-negative individuals could be managed with regular follow-up visits and/or close surveillance. Microecological assessment (e.g., Lactobacillus depletion or Gardnerella enrichment) might be considered as an exploratory adjunct to refine risk stratification in ambiguous cases, but its use remains investigational and requires prospective validation.

Strengths of this study included its prospective design, consecutive enrollment of colposcopy-referred women, and histopathologic confirmation by two independent blinded pathologists, which collectively reduce selection and measurement biases. This investigation had several limitations. The single-center cohort design might introduce selection bias, and limited number of HPV-negative CIN2 + cases, which restricts the ability to explore the interaction between microecological and HPV status, potentially limiting the generalizability of findings. Importantly, our study enrolled women referred for colposcopy following abnormal screening tests or clinical symptoms; therefore, the cohort is enriched for disease and not directly representative of a general screening population, introducing referral bias. As such, the prevalence of disease may be higher and then the performance metrics (sensitivity, specificity, AUC) reported here are influenced by the high pre-test probability of this referral cohort and differ from those obtained in community-based, primary screening populations (e.g., unselected hrHPV-positive women), limiting external validity. The cross-sectional nature of the study precludes inference of temporal or causal relationships between vaginal microecological changes and DNA methylation due to the absence of longitudinal follow-up; thus, it remains unclear the cause-and-effect relationship among dysbiosis, epigenetic changes and HPV infection. Furthermore, financial limitations restricted comprehensive high-throughput microbiome sequencing, with analyses conducted only on a subset of participants during routine outpatient evaluations, which may have lower resolution. Due to the difficulty in collection of clinical data, information on potential confounders such as smoking status, sexual behavior, and parity was not available for all participants; therefore, we could not adjust for these factors, and residual confounding cannot be ruled out. Consequently, our findings should be considered hypothesis-generating with respect to causality or synergistic pathogenesis between methylation and vaginal dysbiosis.

Prospective, population-based studies and longitudinal sampling are needed to determine whether vaginal microecology alterations precede methylation changes or vice versa, or both are parallel consequences of persistent HPV infection. Future work should prioritize multi-center cohorts to validate the CISCER assay for population-based screening in diverse settings, as well as prospective longitudinal cohorts to evaluate whether PAX1/JAM3 methylation predicts lesion progression or regression. Additionally, studies integrating methylation testing into existing HPV screening algorithms—assessing cost-effectiveness and conducting modeling analyses—are recommended. In parallel, high-resolution microbiome and host methylation mechanistic studies are essential to clarify causal relationships and identify potential therapeutic strategies. We believe that concrete future research directions would add value and context to the current findings.

Conclusions

The *PAX1*/*JAM3* methylation test, the CISCER assay, demonstrates robust diagnostic utility for detecting high-grade cervical lesions (CIN2+) and associates with vaginal dysbiosis biomarkers, including *Gardnerella* enrichment and *Lactobacillus* depletion, in colposcopy-referred cohorts. These findings underscore CISCER assay's value as a molecular triage tool for stratifying hrHPV-positive women, potentially reducing unnecessary colposcopy referrals by objectively identifying high-risk cases. The concurrent presence of PAX1/JAM3 hypermethylation and dysbiosis of the vaginal flora suggests that the combined epigenetic-microbial profiling with relevant clinical tests could enhance diagnostic precision, inform therapeutic decisions (e.g., prioritizing intervention or monitoring), and improve prognostic evaluation in CC prevention protocols. Future studies should validate this integrated approach in longitudinal cohorts to establish causal pathways and clinical utility.

Abbreviations

CC	Cervical cancer
CIN	Cervical intraepithelial neoplasia
hrHPV	High-risk human papillomavirus
JAM3	Junctional adhesion molecule 3
PAX1	Paired box gene 1
CISCER	PAX1/JAM3 methylation assay
TZ	Transformation zone

TCT	ThinPrep cytology test
LBC	Liquid-based cytology
ASC-US	Atypical squamous cells of undetermined significance
LSIL	Low-grade squamous intraepithelial lesion
HSIL	High-grade squamous intraepithelial lesion
SCC	Squamous cell carcinoma
AD	Adenocarcinoma
AUC	Area under the curve
NPV	Negative predictive value
PPV	Positive predictive value
AV	Aerobic vaginitis
NMPA	National Medical Products Administration
ROC	The receiver operating characteristic
95% CI	95% confidence intervals

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12905-026-04285-6.

Supplementary Material 1.

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Not applicable.

Authors' contributions
Lianmei Luo and Jun Zhang conceived and designed the work. Lanruo Wang and Zhiying Liu collected the clinical data. Lianmei Luo and Youqin Cai conducted the experiments. Lianmei Luo and Youqin Cai analyzed the data. Lianmei Luo drafted the manuscript.Jun Zhang revised the manuscript. All authors approved the final version.

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Data availability
The original contributions presented in the study were included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Beijing Anzhen Hospital, Capital Medical University (Approval No. KS2023085). All women enrolled in this study provided written informed consent to participate.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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