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# The performance of *JAM3/PAX1* methylation in the diagnosis of high-grade squamous intraepithelial lesions for women with high-risk HPV infection

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## Abstract

**Objective** To assess the clinical value of DNA methylation measurement in exfoliated cervical cells for distinguishing high-grade squamous intraepithelial lesions (HSIL) from other cervical abnormalities.

**Methods** A total of 276 patients were enrolled, and general clinical information was collected. Exfoliated cervical cells were obtained to assess human papillomavirus (HPV) infection, conduct ThinPrep cytology tests (TCT), and measure methylation levels of *JAM3* ( $\Delta$ CtJ) and *PAX1* ( $\Delta$ CtP). Logistic regression was performed to identify factors significantly associated with HSIL diagnosis. A conditional inference tree model and the area under the curve (AUC) were employed to evaluate the efficacy of *JAM3* and *PAX1* methylation in detecting HSIL.

**Results** Independent risk factors for HSIL diagnosis included  $\Delta$ CtJ,  $\Delta$ CtP, atypical squamous cells of undetermined significance (ASCUS), and HPV16 infection. The conditional inference tree indicated that 96.4% of patients were non-HSIL when  $\Delta$ CtJ > 11.66, and 99.1% were non-HSIL when  $\Delta$ CtP > 10.97. The diagnostic performance of  $\Delta$ CtJ/ $\Delta$ CtP surpassed that of TCT/HPV alone. Among six methods, the combination of  $\Delta$ CtP, TCT, and high-risk HPV (hr-HPV) testing achieved the highest sensitivity (91.2%), positive predictive value (50.0%), negative predictive value (98.6%), and AUC (0.932).

**Conclusion** In women with hr-HPV infection, DNA methylation analysis of cervical cytology outperformed traditional TCT or HPV testing. The combination of  $\Delta$ CtP with TCT and HPV may offer the most accurate screening approach for HSIL.

**Keywords** High-grade squamous intraepithelial lesions, DNA methylation, *JAM3*, *PAX1*, Hr-HPV

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## Introduction

Cervical cancer is one of the most prevalent malignancies among women worldwide, particularly in developing countries [1]. The rising mortality rates in developing areas such as Eastern Europe, Central Asia, and Africa are attributed to limited access to effective screening methods and low vaccination coverage [2, 3]. Early detection and intervention of cervical cancer, including screening for high-grade squamous intraepithelial lesions (HSIL), are crucial for reducing the incidence of invasive cervical cancer and improving the prognosis of patients [4]. However, efficient and effective screening methods remain lacking.

Persistent infection with high-risk human papillomavirus (hr-HPV) is the primary cause of cervical intraepithelial neoplasia (CIN) and cervical cancer [5]. While hr-HPV testing is highly sensitive for cervical cancer screening, it suffers from poor specificity [6, 7]. The liquid-based Thinprep cytological test (TCT) is traditionally used in screening, but their relatively low sensitivity and subjectivity, particularly in detecting atypical squamous cells of undefined significance (ASCUS) [8, 9], limit their effectiveness for guiding clinical decisions. However, next-generation sequencing (NGS) has become an effective approach to assess HPV integration status, in which HPV E6/E7 oncogenes are inserted into the host genome. Many studies demonstrate that HPV integration might be a promising triage strategy for HPV-positive patients. Compared with cytology, HPV integration exhibits promising sensitivity and specificity for the diagnosis of CIN grade 3 or more severe (CIN3+) [10, 11]. Although it could help avoid excessive use of invasive cervical biopsies, this NGS-based screening method is resource-intensive and expensive, limiting its application in low-resource area.

Recent studies have noted that cervical cancer pathogenesis is strongly related to the role of epigenetic changes, particularly DNA methylation in cervical cancer development. DNA methylation is a common epigenetic modification and holds promise as a biomarker for cervical cancer diagnosis [12–14]. Detecting DNA methylation levels in key genes during early-stage cervical lesions could facilitate the early identification of high-grade lesions, enabling timely clinical intervention and potentially preventing invasive cervical cancer, such as *ASCL1/LHX8* methylation or WID-qCIN test [15–17]. Notably, the Junctional Adhesion Molecule 3 (*JAM3*) gene has been found to demonstrate higher specificity and a superior positive predictive value in hr-HPV positive patients compared to cytology, suggesting that *JAM3* methylation could serve as a distinguishing marker for such patients [18]. Furthermore, the methylation level of the Paired Box 1 (*PAX1*) gene has been shown to correlate with the

increasing likelihood of cervical lesions and to outperform hr-HPV genotyping alone in diagnostic accuracy [19–22]. However, the ability of *JAM3* or *PAX1* methylation levels in the classification and diagnosis of HSIL remains unclear, particularly in combination of other routine tests including TCT and HPV tests.

Hence, we aimed to evaluate the clinical value of *JAM3* and *PAX1* methylation levels in diagnosing HSIL, comparing their diagnostic accuracy with that of TCT and HPV testing. Most of all, we further developed a more predictive model using a tree-based machine learning method, the conditional tree model, which was chosen due to its direct decision rules for stratification. Its visual representation is directly interpretable, and it can be easily implemented in a clinical setting, particularly for recursive binary splitting [23].

## Materials and methods

### Patients

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Third Xiangya Hospital of Central South University (No. 23137). All participants provided written informed consent. During August–November 2022, 276 women diagnosed with reproductive tract hr-HPV infection at the Third Xiangya Hospital of Central South University were enrolled in this study. A total of 242 patients were pathologically diagnosed with non-HSIL including chronic cervicitis, CIN1, and 34 patients were pathologically diagnosed with HSIL, including CIN2-3.

The subject inclusion criteria were as follows: (1) no vaginal medications within 3 months before sampling; (2) no sexual activity or other vaginal operations within 2 days before sampling; (3) no symptoms or signs of genital tract infection; (4) availability of sexual history; (5) signing the informed consent form. The exclusion criteria were as follows: (1) having received physical therapy and surgery for cervical disease within 3 months; (2) serious heart, liver, kidney, blood system, and autoimmune diseases; (3) incomplete data; (4) multiple hr-HPV infections.

### Clinical data collection

The baseline data of patients were collected: (1) general information including age, height, weight, body mass index (BMI), menopause status; (2) TCT results, categorized into HSIL, low-grade squamous intraepithelial lesions (LSIL), ASCUS, and negative for intraepithelial lesion or malignancy (NILM); (3) HPV results, categorized into HPV16, HPV18, HPV52, HPV58, and other infections; (4) cervical transformation zone from colposcopy, categorized into type I, type II, or type III transformation area and postoperative cervix such as post-loop

electrosurgical excision procedure (LEEP); (5) pathological results of cervical biopsy, categorized into high-grade lesions, low-grade lesions, and chronic cervicitis. The data were carefully recorded and blindly reviewed by two research assistants.

#### DNA methylation detection

A special plastic brush was used to collect exfoliated cells from the surface of the cervix and from inside the cervical canal by rotating 5 times in a clockwise manner, and the sample was placed into a vial containing cell-fixative solution labeled with the detection barcode. The samples were then stored at  $-20^{\circ}\text{C}$  away from light. DNA methylation detection was performed within 1 month after sampling. The DNA methylation of samples was measured by a commercial DNA methylation detection kit (Beijing OriginPoly Bio Tec Co., Ltd.). First, a JH-DNA separation and purification kit (Spin Column) was used to extract DNA from exfoliated cervical cells, and DNA concentration and quality were determined. Subsequently, the DNA samples were treated with sulfite. Multiple real-time quantitative PCR technology (SLAN-96S Automatic Medical PCR Analysis System) was applied to detect the methylation status of the target *PAX1* and *JAM3* genes, while *GAPDH* was adopted as the internal reference gene. The reaction conditions were as follows: predenaturation at  $96^{\circ}\text{C}$  for 10 min; 45 cycles of denaturation at  $94^{\circ}\text{C}$  for 15 s and annealing at  $64^{\circ}\text{C}$  for 5 s; extension; fluorescence acquisition at  $60^{\circ}\text{C}$  for 30 s. The instrument was cooled at  $25^{\circ}\text{C}$  for 1 min. After the reaction, the Ct values of *JAM3* ( $\Delta\text{CtJ}$ ), *PAX1* ( $\Delta\text{CtP}$ ), and internal reference gene ( $\Delta\text{Ct} = \text{Ct detection gene} - \text{Ct internal reference gene}$ ) were calculated.

#### TCT

The sampling method for TCT was identical to that used for DNA methylation detection. Samples were immediately rinsed in a cell preservation solution vial (Hologic, Inc., Massachusetts, United States) by pushing the collection brush into the vial 10 times, ensuring that the bristles were forced apart. The exfoliated cells in the solution were processed onto glass slides using the ThinPrep 2000 processor. The slides were then fixed with 95% ethanol and stained. Cytopathologists examined the slides under a microscope, and diagnoses were made according to the revised 2014 cervical cytological grading system [24].

#### HPV detection

HPV genotyping test was performed as previously described [25]. Briefly, cervical cells were collected by gently scraping the cervix with a brush, and DNA was extracted using the Magnetic Beads DNA Purification Kit (HybriBio Ltd., Chaozhou, China) according to the

manufacturer's instructions. HPV DNA was amplified using PCR, and genotyping was conducted with the HPV GenoArray Test Kit (HybriBio Ltd., Chaozhou, China). This test detected 21 HPV types, including 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), 5 low-risk types (6, 11, 42, 43, and 44), and 2 types of uncertain risk (53 and CP8304) [26].

#### Colposcopy and biopsy

A colposcopy was performed when cervical cancer screening results indicated abnormal cellular changes or hr-HPV positivity. A speculum was inserted to hold apart the vaginal walls, allowing visualization of the cervix and vagina. A Leisegang 3ML colposcope (Leisegang, Berlin, Germany) was placed near the vaginal opening, and 5% acetic acid and Lugol's iodine solutions were applied to detect lesions. Transformation zones were classified as type I, II, or III, depending on visibility [27]. Patients with previous cervical resection surgery or LEEP procedures were considered postoperative. Cervical biopsies were taken from suspicious lesions, with tissue samples fixed in 10% formalin and submitted for histopathological examination. Diagnoses were made by two certified pathologists through double-blind analysis using optical microscopy. Histopathological results followed WHO guidelines, with CIN1 classified as low-grade lesions and CIN2-3 as high-grade lesions [28]. For this study, a binary classification of HSIL and non-HSIL was used, with low-grade lesions and chronic cervicitis grouped as non-HSIL for statistical analysis.

#### Statistical analysis

The Shapiro–Wilk test was used to assess the normality of each variable. Data with a normal distribution were expressed as means  $\pm$  standard deviations, while non-normally distributed data were presented as medians (P25, P75). The independent two-sample t-test or Mann–Whitney U test was applied to analyze continuous variables, while the  $\chi^2$  test or Fisher's exact test was used for categorical variables. Univariate and multivariate logistic regression analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). Correlation analysis was conducted using the Pearson test for continuous variables and the Spearman test for categorical variables. A conditional inference tree was constructed using R version 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria). The area under the curve (AUC) was employed to evaluate the diagnostic performance of each index for high-grade cervical lesions. A *P*-value of  $<0.05$  was considered statistically significant.

## Results

### Clinical characteristics of patients

A total of 276 patients met the inclusion criteria and were analyzed. Based on cervical biopsy histopathology, patients were divided into two groups: non-HSIL ( $n=242$ ) and HSIL ( $n=34$ ). Table 1 summarized the comparison of clinical characteristics between these groups. No significant differences were observed in age or BMI. However, the methylation levels of *JAM3* and *PAX1* were significantly lower in the non-HSIL group compared to the HSIL group, indicating distinct epigenetic profiles between the groups and consistent with previous reports [29, 30].

### Univariate and multivariate logistic regression analysis of risk factors for HSIL

Univariate logistic regression analysis was performed to identify risk factors associated with HSIL. The analysis revealed that  $\Delta\text{CtP}$ ,  $\Delta\text{CtJ}$ , TCT\_HSIL, TCT\_ASCUS, HPV16 infection, HPV52 infection, and HPV58 infection were independent risk factors for HSIL (Table 2).

Next, multivariate logistic regression analysis was conducted on variables with  $P<0.05$ . Due to collinearity between  $\Delta\text{CtP}$  and  $\Delta\text{CtJ}$ , they were analyzed separately to avoid issues such as inaccurate coefficient estimates,

difficulty in model interpretation, and reduced predictive power. The results showed that  $\Delta\text{CtP}$ ,  $\Delta\text{CtJ}$ , ASCUS, and HPV16 infection were independent risk factors for HSIL ( $P<0.05$ ).

### Correlation analysis

Correlation analysis indicated a negative correlation between cervical pathology results and  $\Delta\text{CtP}$  ( $r=-0.447$ ,  $P<0.001$ ),  $\Delta\text{CtJ}$  ( $r=-0.532$ ,  $P<0.001$ ), TCT ( $r=-0.179$ ,  $P=0.003$ ), and HPV ( $r=-0.268$ ,  $P<0.001$ ). Among these,  $\Delta\text{CtP}$  and  $\Delta\text{CtJ}$  showed stronger correlation indices, suggesting a relatively stronger association. No significant correlations were found between cervical pathology results and age, BMI, menopausal status, or transformation zone type ( $P<0.05$ , Fig. 1).

### Conditional inference tree model

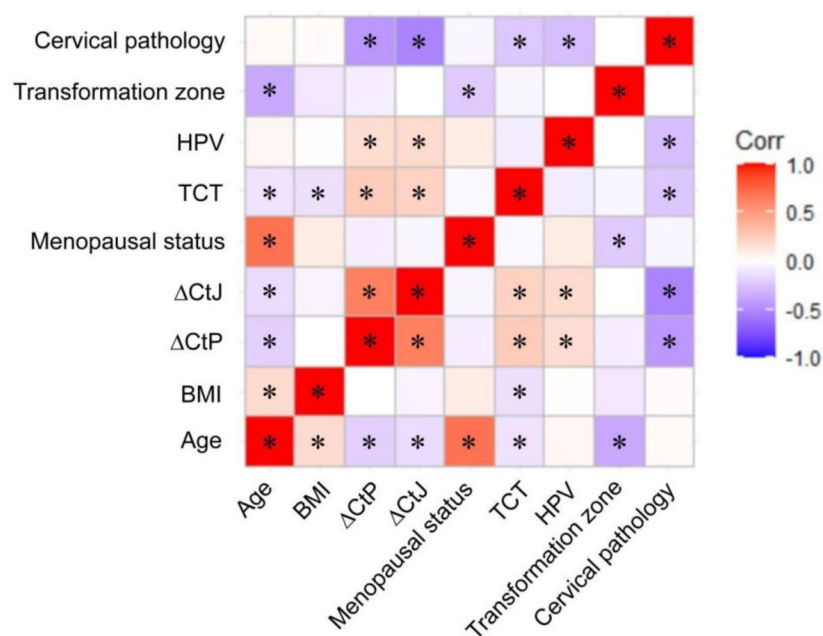
To evaluate the predictive ability of *JAM3* and *PAX1* gene methylation levels for HSIL diagnosis, the variables  $\Delta\text{CtJ}$ ,  $\Delta\text{CtP}$ ,  $\Delta\text{CtJ}+\text{TCT}+\text{HPV}$ , and  $\Delta\text{CtP}+\text{TCT}+\text{HPV}$  were included in the conditional inference tree model (Fig. 2), based on the univariate and multivariate logistic regression analyses. And the cutoff value was determined by the Classification And Regression Tree (CART) algorithm. Due to the

**Table 1** Clinical characteristics of the study participants

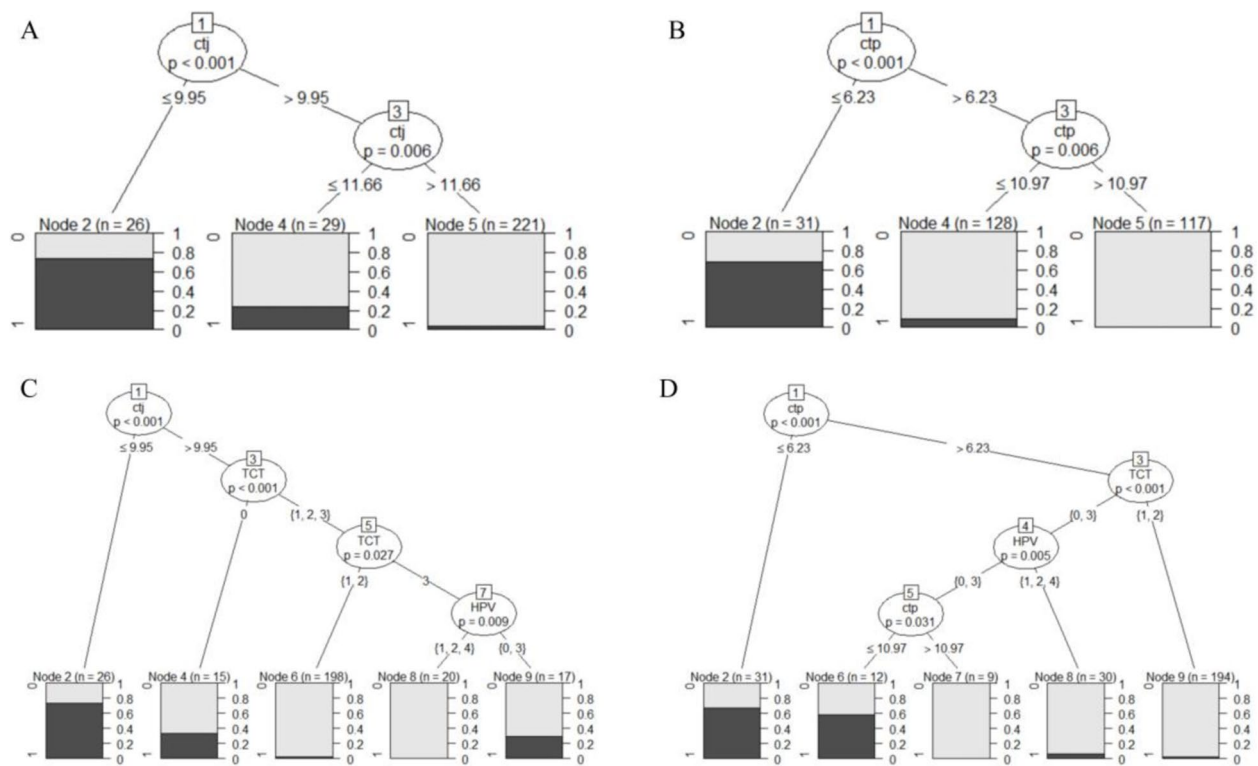
Variables	Total ( $n=276$ )	non-HSIL group ( $n=242$ )	HSIL group ( $n=34$ )
Age /y(range)	38.50(32.0, 50.0)	38.00(31.0, 50.0)	39.50(33.0, 50.0)
BMI /Kg/m <sup>2</sup>	21.50(19.6, 23.8)	21.50(19.6, 23.8)	21.50(19.9, 23.8)
$\Delta\text{CtP}$	10.32(8.18, 16.53)	10.83(8.85, 16.88)	5.62(2.97, 8.05)
$\Delta\text{CtJ}$	14.69(12.32, 15.91)	14.94(13.53, 16.03)	9.59(6.60, 11.66)
Menopause			
No	207 (75.00)	180 (74.38)	27 (79.41)
Yes	69 (25.00)	62 (25.62)	7 (20.59)
TCT			
HSILs	27 (9.78)	12 (4.96)	15 (44.12)
LSILs	62 (22.46)	57 (23.55)	5 (14.71)
ASCUS	146 (52.90)	140 (57.85)	6 (17.65)
NILM	41 (14.86)	33 (13.64)	8 (23.52)
HPV			
16	61 (22.10)	42 (17.36)	19 (55.88)
18	20 (7.25)	20 (8.26)	0 (0)
52	67 (24.28)	60 (24.79)	7 (20.59)
58	45 (16.30)	38 (15.70)	7 (20.59)
Others	83 (30.07)	82 (33.89)	1 (2.94)
Colposcopy results			
Type III	116 (42.03)	102 (42.15)	14 (37.84)
Type II	24 (8.70)	22 (9.09)	2 (5.88)
Type I	116 (42.03)	99 (40.91)	17 (50.00)
Postoperation	20 (7.25)	19 (7.85)	1 (6.28)

**Table 2** Univariate and multivariate logistic regression analyses of the clinical indicators related to HSIL

Variables	Univariate		Multivariate ( $\Delta$ CtP)		Multivariate ( $\Delta$ CtJ)	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
<b>Age (y)</b>	1.01 (0.98–1.04)	0.651	-	-	-	-
<b>BMI (Kg/m<sup>2</sup>)</b>	1.01 (0.94–1.08)	0.693	-	-	-	-
<b>Menopause</b>						
No	1.33 (0.55–3.20)	0.527	-	-	-	-
Yes	-	-	-	-	-	-
<b><math>\Delta</math>CtP</b>	0.63 (0.53–0.72)	< 0.001	0.68 (0.58–0.81)	< 0.001	-	-
<b><math>\Delta</math>CtJ</b>	0.62 (0.53–0.71)	< 0.001	-	-	0.7 (0.6–0.81)	< 0.001
<b>TCT</b>						
HSILs	5.16 (1.75–15.23)	0.003	1.85 (0.37–9.11)	0.452	2.35 (0.51–10.82)	0.274
LSILs	0.36 (0.11–1.20)	0.096	0.25 (0.06–1.14)	0.073	0.36 (0.08–1.53)	0.165
ASCUS	0.18 (0.06–0.54)	0.003	0.16 (0.04–0.66)	0.012	0.17 (0.04–0.67)	0.012
NILM	-	-	-	-	-	-
<b>HPV</b>						
16	37.10(4.80–286.71)	0.005	9.91 (1.12–87.36)	0.039	11.24 (1.3–96.94)	0.028
18	< 0.01 (< 0.01–> 999.99)	0.981	< 0.01 (< 0.01–> 999.99)	0.976	< 0.01 (< 0.01–> 999.99)	0.977
52	9.57 (1.15–79.82)	0.037	2.14 (0.21–21.79)	0.520	2.87 (0.29–28.54)	0.368
58	15.11(1.80–127.15)	0.013	4.99 (0.51–48.76)	0.167	7.02 (0.75–65.97)	0.088
Others	-	-	-	-	-	-
<b>Colposcopy results</b>						
Type III	-	-	-	-	-	-
Type II	0.66 (0.10–2.60)	0.603	-	-	-	-
Type I	1.25 (0.59–2.71)	0.563	-	-	-	-
Postoperation	0.38 (0.02–2.09)	0.368	-	-	-	-

**Fig. 1** Heatmap depicting the correlation between different clinical variables. \* $P < 0.05$





**Fig. 2** Predictors for the hierarchical diagnosis of HSIL using conditional inference tree. **A**  $\Delta CtJ$ : (1)  $\leq 9.95$ , 26.9% of patients were non-HSIL; (2)  $> 9.95$  and  $\leq 11.66$ , 75.9% of patients were non-HSIL; (3)  $> 11.66$ , 96.4% of patients were non-HSIL. **B**  $\Delta CtP$ : (1)  $\leq 6.23$ , 32.3% of patients were non-HSIL; (2)  $> 6.23$  and  $\leq 10.97$ , 90.6% of patients were non-HSIL; (3)  $> 10.97$ , 99.1% of patients were non-HSIL. **C**  $\Delta CtJ$  + TCT + HPV: (1)  $\Delta CtJ \leq 9.95$ , 26.9% of patients were non-HSIL; (2)  $\Delta CtJ > 9.95$  and a TCT result of HSIL: 66.7% of patients were non-HSIL; (3)  $\Delta CtJ > 9.95$  and a TCT result of LSILs/ASCUS: 97.5% of patients were diagnosed with non-HSIL; (4)  $\Delta CtJ > 9.95$ , a TCT result of NILM, and an HPV strain of 18/52/others: all patients were non-HSIL; (5)  $\Delta CtJ > 9.95$ , a TCT result of NILM, and an HPV strain of 16/58: 70.6% of patients were non-HSIL. **D**  $\Delta CtP$  + TCT + HPV: (1)  $\Delta CtP \leq 6.23$ , 32.3% of patients were non-HSIL; (2)  $\Delta CtP > 6.23$  and a TCT result of LSILs/ASCUS: 97.9% of patients were non-HSIL; (3)  $\Delta CtP > 6.23$  with a TCT result of HSILs/NILM and an hr-HPV infection of strains 18/52/others: 93.3% of patients were non-HSIL; (4) a TCT result of HSIL/NILM, HPV infection of strains 18/52/others and  $\Delta CtP > 6.23$  but  $\leq 10.97$ : 41.7% of patients were non-HSIL; (5)  $\Delta CtP > 10.97$ , a TCT result of HSIL/NILM and hr-HPV infection of strains 18/52/others: all of patients were non-HSIL

collinearity between  $\Delta CtP$  and  $\Delta CtJ$ , it was inappropriate to construct a model that included both variables simultaneously.

Figure 2A showed a tree divided into two layers, producing three classification rules based on  $\Delta CtJ$  levels: 1)  $\Delta CtJ \leq 9.95$ : This category included 26 patients, of whom 7 (26.9%) were classified as non-HSIL, while 19 (73.1%) were diagnosed with HSIL. 2)  $\Delta CtJ > 9.95$  and  $\leq 11.66$ : This category contained 29 people, with 22 (75.9%) classified as non-HSIL, indicating an increase in the proportion of non-HSIL cases compared to the first category. 3)  $\Delta CtJ > 11.66$ : This group contained 221 patients, of whom 96.4% were patients with non-HSIL, while only 3.6% were patients with HSIL. This model demonstrated that as  $\Delta CtJ$  increased, the likelihood of HSIL decreased. When  $\Delta CtJ$  exceeded 11.66, the probability of HSIL was very low, indicating that this value may serve as an effective cutoff for distinguishing HSIL from non-HSIL based on JAX3 methylation levels.

Figure 2B presented a similar tree model based on  $\Delta CtP$ , also divided into two layers with three classification rules: 1)  $CtP \leq 6.23$ : This category included 31 patients, with 10 (32.3%) classified as non-HSIL and 21 (67.7%) diagnosed with HSIL. 2)  $\Delta CtP > 6.23$  and  $\leq 10.97$ : This second category consisted of 128 patients, with 116 (90.6%) classified as non-HSIL, representing a significant increase in non-HSIL cases compared to the first category. 3)  $\Delta CtP > 10.97$ : This group comprised 117 patients, with almost all classified as non-HSIL, and only 0.9% diagnosed with HSIL. Similar to the model in Fig. 2A, this tree indicated that as  $\Delta CtP$  increased, the probability of HSIL decreased. When  $\Delta CtP$  exceeded 10.97, non-HSIL could be distinguished easily, with a very low likelihood of HSIL.

We further evaluated the ability of DNA methylation levels, combined with traditional TCT/HPV screening methods, to distinguish patients with HSIL. The conditional inference tree in Fig. 2C included  $\Delta CtJ$ , TCT, and

**Table 3** Accuracy of relevant indicators for screening HSIL

Indicators	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)
TCT	0.676	0.814	0.338	0.947	0.791 (0.703,0.880)
HPV	0.765	0.669	0.245	0.953	0.784 (0.717,0.851)
$\Delta$ CtP	0.765	0.831	0.388	0.962	0.867 (0.804,0.931)
$\Delta$ CtJ	0.765	0.880	0.473	0.964	0.841 (0.750,0.932)
$\Delta$ CtP + TCT + HPV	0.912	0.872	0.500	0.986	0.932 (0.881,0.983)
$\Delta$ CtJ + TCT + HPV	0.882	0.847	0.448	0.981	0.926 (0.876,0.975)

HPV: 1)  $\Delta$ CtJ  $\leq$  9.95: This category included 26 patients, with results identical to those described in Fig. 2A. 2)  $\Delta$ CtJ  $>$  9.95 and a TCT result of HSIL: This second category included 15 patients. The proportion of non-HSIL increased from 26.9% to 66.7%, while HSIL accounted for 33.3%. 3)  $\Delta$ CtJ  $>$  9.95 and a TCT result of LSILs or ASCUS: This category consisted of 198 patients, of whom 97.5% were diagnosed with non-HSIL. 4)  $\Delta$ CtJ  $>$  9.95, a TCT result of NILM, and an HPV strain of 18, 52, or others: This category comprised 20 patients, all of whom were diagnosed with non-HSIL. 5)  $\Delta$ CtJ  $>$  9.95, a TCT result of NILM, and an HPV strain of 16 or 58: This group included 17 patients, 12 (70.6%) of whom had non-HSIL, while 5 (29.4%) were diagnosed with HSIL, demonstrating a lower predictive performance than the fourth category. These findings indicated that the combination of  $\Delta$ CtJ with TCT and HPV did not significantly enhance the predictive ability for HSIL compared to  $\Delta$ CtJ or  $\Delta$ CtP alone (Fig. 2A, B). In other words, combining  $\Delta$ CtJ with TCT and HPV was not superior to using  $\Delta$ CtJ or  $\Delta$ CtP independently.

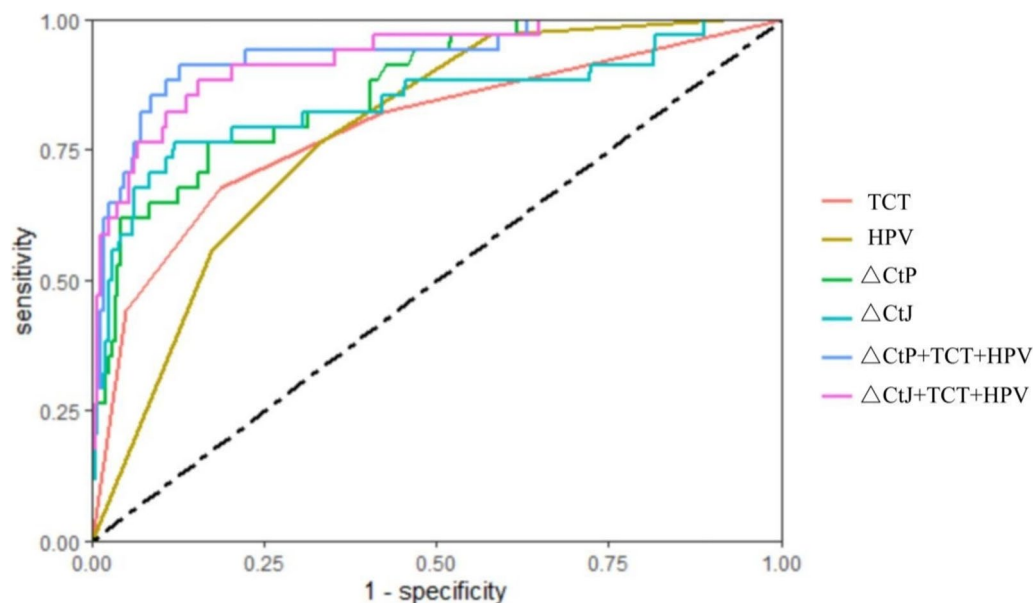
The conditional inference tree in Fig. 2D incorporated  $\Delta$ CtP, TCT, and HPV: 1)  $\Delta$ CtP  $\leq$  6.23: The results in this category mirrored those in Fig. 2B. 2)  $\Delta$ CtP  $>$  6.23 and a TCT result of LSILs/ASCUS: This category included 194 patients, with 97.9% diagnosed as non-HSIL. 3)  $\Delta$ CtP  $>$  6.23 with a TCT result of HSILs/NILM and an hr-HPV infection of strains 18/52/others: This category consisted of 30 patients, 93.3% of whom were diagnosed with non-HSIL. 4) a TCT result of HSIL/NILM, HPV infection of strains 18/52/others and  $\Delta$ CtP  $>$  6.23 but  $\leq$  10.97: In this category of 12 patients, 41.7% were diagnosed as non-HSIL, and the differentiation between HSIL and non-HSIL was not optimal. 5)  $\Delta$ CtP  $>$  10.97, a TCT result of HSIL/NILM and hr-HPV infection of strains 18/52/others: This group included 9 patients, all

of whom were diagnosed with non-HSIL. This model demonstrated that  $\Delta$ CtP combined with TCT and HPV provided good predictive ability for HSIL and effectively distinguished between the two patient groups.

#### Clinical efficacy of single and combined parameters in the diagnosis of high-grade cervical lesions

Based on the results of the conditional inference tree, cutoff values of 9.95 and 6.23 were selected as optimal for diagnosing HSIL using  $\Delta$ CtJ and  $\Delta$ CtP, respectively. To further evaluate the predictive value of different models for HSIL diagnosis, we compared the clinical efficacy and AUC values of six models (Table 3). We found that: (1) the combination of  $\Delta$ CtP with TCT + HPV demonstrated the highest clinical efficacy, with a sensitivity of 91.2%, specificity of 87.2%, a positive predictive value (PPV) of 50.0%, and a negative predictive value (NPV) of 98.6%; (2) the second-best model was  $\Delta$ CtJ combined with TCT + HPV; (3) the clinical efficacy of  $\Delta$ CtP and  $\Delta$ CtJ alone was comparable, ranking just below their combination with TCT and HPV; (4) the efficacy of TCT or HPV alone was the lowest; (5) the combination of  $\Delta$ CtJ + TCT + HPV showed no significant advantage over the single application of  $\Delta$ CtJ. While it improved sensitivity (88.2% vs. 76.5%) and NPV (98.1% vs. 96.4%), it slightly reduced specificity (84.7% vs. 88.0%) and PPV (44.8% vs. 47.3%).

The ROC curve for each clinical index in diagnosing HSIL was shown in Fig. 3. The AUC value for  $\Delta$ CtP combined with TCT + HPV was the highest at 0.932. The model incorporating  $\Delta$ CtJ combined with TCT + HPV ranked second, with an AUC value of 0.926. The AUC values for  $\Delta$ CtP and  $\Delta$ CtJ alone were 0.867 and 0.841, respectively, both lower than those of the combined models. The AUC values for TCT and HPV alone were the lowest, at 0.791 and 0.784, respectively.



**Fig. 3** ROC curve of each clinical index for HSIL diagnosis

## Discussion

Hypermethylation of tumor cell-specific gene promoters is recognized as an epigenetic mechanism that facilitates tumor occurrence [31]. Hypermethylation of the gene promoter primarily occurs within the CpG islands of promoter regions and their adjacent areas, leading to reduced gene expression without altering the protein-coding sequence. As a result, gene silencing and dysregulation of signal transduction pathways occur, contributing to abnormal gene expression [31, 32]. In terms of epigenetic modification of cervical cancer, studies reported specific DNA methylation profiles in tumor suppressor genes, highlighting the potential utility of gene hypermethylation as a biomarker for early diagnosis of precancerous cervical lesions and cervical cancer [33].

*JAM3* is a transmembrane protein that belongs to the immunoglobulin superfamily. Boers et al. evaluated the clinical utility of *JAM3* gene methylation in diagnosing CIN2 or worse in women with hr-HPV infection using quantitative methods. They found that *JAM3* methylation had a sensitivity of 68% and specificity of 94% in all patients, while the sensitivity and specificity for diagnosing CIN3 or worse were 80% and 76%, respectively [34]. In another study by the same group, *JAM3* exhibited high sensitivity and specificity (82% and 88%, respectively) in diagnosing CIN3+ lesions from hr-HPV positive samples collected by physicians. By comparison, cytology showed a sensitivity of 91% but a lower specificity of 48% [35]. In our study, we found that the efficacy of HSIL using  $\Delta CtJ$  alone diminished as the cutoff value increased. When

$\Delta CtJ$  exceeded 11.66, 96.4% of patients were diagnosed as non-HSIL, with only 3.6% confirmed as HSIL, demonstrating a strong ability to differentiate between HSIL and non-HSIL. However, the sensitivity of the conditional inference tree model for diagnosing HSIL was 76.5%, lower than previous reports. This discrepancy may be due to the inclusion of both hr-HPV and non-hr-HPV-infected individuals in our study, which better reflects real-world clinical application.

*PAX1* encodes a conserved region related to transcription factors and plays a critical role in embryonic development [36]. Liu et al. [37] found that *PAX1* gene methylation is prevalent in exfoliated cervical cells from patients with grade III cervical intraepithelial lesions and invasive cervical cancer. Xu et al. [38] reported that while the gene was not methylated in normal cervical cells, methylation occurred in 9% of CIN1 cases, 44% in CIN2/3, and 100% in invasive cervical cancer cases. Our findings showed that the cutoff for  $\Delta CtP$  was 6.23, closely aligning with the 6.6 value used in the cervical cancer DNA methylation detection kit (Beijing Origin-Poly Bio Tec Co., Ltd.). When  $\Delta CtP$  alone was used with a cutoff of 10.97, 99.3% of patients were non-HSIL, and only 0.9% were diagnosed with HSIL. The diagnostic accuracy of  $\Delta CtP$  in distinguishing HSIL from non-HSIL was comparable to manual TCT and consistent with previous studies [39].

The sensitivity and specificity of TCT vary significantly depending on the medical expertise in different



regions [40]. A notable proportion of HSIL is missed when patients are diagnosed with ASCUS [41, 42]. Numerous studies suggest that DNA methylation testing exhibits higher specificity than cytology and greater sensitivity than HPV16/18 genotyping in patients with ASCUS, making it a promising approach for cervical cancer screening [43]. Additionally, several studies [44–46] have shown that methylation markers are more sensitive than protein markers, as methylation changes often occur in the early stages of cancer. Our study demonstrated that  $\Delta$ CtP with TCT and HPV provided the optimal model for diagnosing HSIL, achieving a better sensitivity of 91.2%, a PPV of 50.0%, an NPV of 98.6%, an AUC of 0.932 and a compatible specificity of 87.2%, indicating that incorporating  $\Delta$ CtP detection improves the accuracy of HPV and TCT in predicting high-grade lesions.

In addition to sensitivity and specificity, traditional cytology tests and hr-HPV screening presented several limitations, particularly for large-scale cervical cancer screening worldwide. Conventional cytology tests, such as TCT, require highly skilled cytopathologists, making the process labor-intensive and subjective [47]. In low-income countries, hr-HPV genotyping is constrained by limited resources, including a shortage of trained personnel and advanced equipment. Furthermore, using hr-HPV testing alone for cervical cancer screening is challenging because only a small percentage of HPV-infected individuals, even those with hr-HPV, will develop cancer [48]. HPV integration test could help to distinguish these high-risk patients but requires intensive resources. In contrast, DNA methylation testing offers more objective and efficient results, as it is quantitative and relative cheap. Additionally, DNA methylation samples can be self-collected at home, with accuracy comparable to those collected by specialized gynecologists [35].

However, this study had several limitations. First, the sample size was small, leading to biased or misleading outcomes and necessitating larger-scale prospective studies to validate the findings. And due to the small size of patients, we were unable to do an external validation, which would help to convince readers or researchers that *JAM3/PAX1* methylation test could be a promising triage marker for cervical cancer screening. Second, while the conditional inference tree showed strong predictive ability, it could not quantify the risk associated with each variable. Additionally, the methylation levels of *JAM3* and *PAX1* could not be combined into a single model due to its collinearity, leaving the combined efficacy of multiple gene methylation levels unclear. Although some statistical approaches, such as principal component analysis and stepwise approaches, could fix the collinearity and

develop more comprehensive predictive models, we are perusing genes whose methylation level could link to HSIL diagnosis in future to establish a much more accurate model.

## Conclusion

This study highlighted that the methylation levels of specific genes, such as *JAM3* and *PAX1*, were promising biomarkers for distinguishing HSIL from non-HSIL. When combined with TCT and HPV testing, these markers improved screening accuracy with better sensitivity and specificity. Mostly, it could prevent unnecessary referrals for colposcopy in population-based screening programs particular in resource-limited areas for women with high-risk HPV infection. Therefore,  $\Delta$ CtJ and  $\Delta$ CtP were valuable auxiliary diagnostic tools for cervical cancer screening, aiding in the early detection of HSIL and potentially reducing the incidence of invasive cervical cancer.

## Abbreviations

ASCUS	Atypical squamous cells of undetermined significance
AUC	The area under the curve
BMI	Body mass index
CIN	Cervical intraepithelial neoplasia
HPV	Human papillomavirus
hr-HPV	High-risk HPV
<i>JAM3</i>	Junctional Adhesion Molecule 3
LEEP	Loop electrosurgical excision procedure
LSIL	Low-grade squamous intraepithelial lesions
NGS	Next-generation sequencing
NILM	Negative for intraepithelial lesion or malignancy
NPV	Negative predictive value
<i>PAX1</i>	Paired Box 1
PPV	Positive predictive value
TCT	ThinPrep cytology tests

## Acknowledgements

Not applicable.

## Authors' contributions

X.Z. and D.X. conceived and designed the study. D.S. and C.S. drafted the manuscript and analyzed the data. F.Z. collected data. All authors contributed to the article and approved the submitted version.

## Funding

None.

## Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. (E-mail: zhaoxing-ping8846@163.com and dabaouxu2022@163.com).

## Declarations

### Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Third Xiangya Hospital of Central South University (No. 23137). All participants provided written informed consent.

### Consent for publication

Not applicable.

# Competing interests

The authors declare no competing interests.

Received: 23 August 2024 Accepted: 5 December 2024

Published online: 18 December 2024

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