Analysis of DNA methylation markers for diagnosing cervical precancer and cancer: A single-center cross-sectional study

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Abstract. Methylation biomarkers of six tumor suppressor genes (astrotactin 1, distal-less homeobox 1, integrin subunit α 4, relaxin family peptide receptor 3, SOX17 and zinc finger protein 671) were evaluated for their diagnostic efficacy in identifying cervical precancerous lesions and cervical cancer (CC). A total of 283 cases were selected from the General Hospital of Ningxia Medical University (Yinchuan, China) between September 2023 and November 2024. Based on histopathologic diagnoses, patients were divided into four groups: The control group, intraepithelial lesions (LSIL) group, high-grade squamous intraepithelial lesions (HSIL) group and CC group. DNA methylation detection, human papillomavirus (HPV) analysis and cytology analysis were performed. The diagnostic value of DNA methylation detection for CC was assessed by calculating the positive methylation rate, sensitivity, specificity, area under the curve (AUC) and other efficacy indicators. The positive methylation rates and methylation scores of DNA methylation in the HSIL and CC groups were significantly higher compared with those in the control and LSIL groups. Combined DNA methylation detection demonstrated the highest diagnostic efficacy for HSIL and CC, with an AUC value of 0.84, a Youden index of 0.69 and a sensitivity and specificity of 82.0 and 87.0%, respectively. In addition, the diagnostic performance of combined detection was significantly higher compared with that of HPV testing and liquid-based cytology testing ($P<0.05$). In addition, the diagnostic efficacy of ZNF671 was significantly higher compared with that of the other five methylation markers, with an AUC value of 0.87, a Youden index of 0.73 and a sensitivity and specificity of 78.0 and 95.0%, respectively. Overall, multiple gene site DNA methylation detection was found to be a valuable diagnostic method for CC and may have potential applications in clinical practice.

Introduction

With an estimated 604,000 new cases and 280,000 mortalities from cervical cancer (CC) in 2024, CC ranks as the fourth most common cause of cancer-associated mortalities among women worldwide (1). In the field of CC and its precursor lesions, DNA methylation, an epigenetic modification, has gradually emerged as a research focus (2,3). CC poses a notable threat to global women's health and is closely associated with human papillomavirus (HPV) infection, particularly HPV16 and HPV18 (4). Currently, CC screening primarily relies on liquid-based cytology (LBC) and high-risk HPV DNA testing (5,6). However, the accuracy and reliability of these diagnostic approaches are limited by subjective factors, including sampling methods, slide preparation techniques and the interpretative abilities of pathologists (7,8). Therefore, it is important to identify effective biomarkers capable of detecting CC at an early stage, thereby improving diagnostic accuracy and treatment outcomes.

DNA methylation is an epigenetic modification process in which DNA methyltransferases catalyze the addition of a methyl group to the 5-carbon position of the cytosine ring, resulting in the formation of 5-methylcytosine (9). This

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modification primarily occurs at CpG dinucleotides, particularly within CpG islands, which are enriched in CpG pairs and typically located in gene promoter regions. Under physiological conditions, these regions are generally unmethylated (10). Aberrant DNA methylation, such as hypermethylation of CpG sites in gene promoter regions, often results in transcriptional silencing, whereas hypomethylation within gene bodies typically leads to increased gene expression (11). DNA methylation has been consistently demonstrated to serve a key role in the development of numerous cancer types, including CC (12,13). Aberrant DNA methylation can influence the expression of tumor suppressor genes and oncogenes. Previous studies have shown that the methylation status of promoter regions in genes such as erythrocyte membrane protein band 4.1 like 3, family with sequence similarity 19 [chemokine (C-C motif)-like] member A4, junctional adhesion molecule 3, microRNA (miR)-124-2, paired box 1 (PAX1), zinc finger protein 671 (ZNF671) and SOX1 is associated with the severity of cervical histopathological lesions (14-17). The methylation status of septin 9 may serve as a promising and reliable biomarker for the identification of CC and as an alternative diagnostic tool in HPV-driven CC screening protocols (18).

A previous study found that astrotactin 1 (ASTN1) expression in hepatocellular carcinoma is associated with tumor immune infiltration and inhibits the migration and invasion of liver cancer cells through the Wnt/β-catenin signaling pathway. The expression level of ASTN1 in liver cancer tissues has been reported to be lower compared with that in matched normal tissues and has been associated with poor prognosis in patients with liver cancer (19). An additional study revealed the oncogenic role of distal-less homeobox 1 (DLX1) in prostate cancer and demonstrated that inhibition of its function reduced tumorigenesis and metastasis (20). Research has also shown that high integrin subunit α 4 (ITGA4) expression is associated with poor prognosis in patients with gastric cancer (21). Hypermethylation of relaxin family peptide receptor 3 (RXFP3) promoter regions appears to be a frequent event in endometrial carcinomas (22). A further study found that SOX17 not only participates in tumor cell proliferation and migration by acting as a negative regulator but also promotes tumor growth by suppressing immune responses (23). Research regarding multi-gene panel detection for cervical precancerous lesions and CC remains relatively limited. A number of epigenetic gene markers, including ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671, have been proposed for the early detection and risk assessment of CC, thereby improving the accuracy and reliability of diagnostic tests (24-27).

The six host gene methylation markers included in the GynTect® assay (ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671) were selected based on systematic bioinformatic evidence, population epidemiological characteristics and previous clinical validation results (26,28). Considering the unique HPV genotype distribution among Chinese women, in which HPV52 and HPV58 are prevalent in addition to HPV16/18 (29,30), traditional triage markers, including cytology and HPV16/18 genotyping, which were developed based on Western populations, may have limited applicability in Chinese populations. Therefore, the six-gene panel was selected due to its stable diagnostic performance across different high-risk HPV subtypes. Furthermore, accumulating clinical evidence and preliminary cohort studies have demonstrated that ZNF671 exhibits good discriminatory capacity for distinguishing high-grade cervical lesions from transient HPV infections, supporting its potential value in the precise triage of HPV-positive patients (31,32).

The present study selected a kit containing six genes (ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671), which have been extensively investigated for gene methylation detection, to explore the application value of multi-gene methylation detection in the diagnosis of CC in China and to provide additional references for clinical practice.

Materials and methods

Study participants. A convenience sampling method was used in the present retrospective study. All eligible participants were consecutively enrolled from patients who visited the General Hospital of Ningxia Medical University (Yinchuan, China) during the present study period. Written informed consent was obtained from all participants prior to data collection. A total of 344 patients who attended the hospital between September 2023 and November 2024 and were referred for colposcopy due to abnormal HPV-DNA or cytological test results were initially included in the present study cohort. All patients subsequently underwent cervical conization or total hysterectomy. As shown in Fig. 1, 283 samples remained eligible for statistical analysis after 61 samples were excluded according to the exclusion criteria and sample quality assessment. Cervical intraepithelial neoplasia (CIN) is a histologically defined premalignant lesion of the cervix, classified into three grades based on the depth of epithelial involvement. CIN1 corresponds to mild dysplasia involving the lower one-third of the epithelium, CIN2 corresponds to moderate dysplasia involving the lower two-thirds and CIN3 corresponds to severe dysplasia or carcinoma *in situ* involving more than two-thirds to the full thickness of the epithelium. Based on histopathological diagnoses independently evaluated by two experienced pathologists, the samples were classified into the control, intraepithelial lesions (LSIL; including CIN1), high-grade squamous intraepithelial lesions (HSIL; including CIN2 and CIN3) and CC groups. The control group consisted of patients who underwent total hysterectomy for benign gynecological diseases unrelated to cervical lesions. Postoperative pathological examination of cervical tissues determined the absence of any cervical epithelial abnormalities, including LSIL, HSIL or cervical carcinoma. Individuals with any cervical pathological abnormalities were excluded from the control group. Data regarding methylation analysis, HPV-DNA testing, cytological examination, colposcopy and clinical characteristics were collected. The inclusion criteria ensured sexual history, HPV-DNA screening results, cervical cytology screening results and cervical lesions were determined by colposcopy-guided biopsy histopathology. The exclusion criteria were as follows: i) Pregnancy, breastfeeding or a history of reproductive system malignancies; ii) vaginal examination or menstruation within 2 days prior to colposcopy; iii) insufficient DNA concentration in the cytological sample or sample loss; and iv) a history of cervical surgery.

Histopathological reference standard. All cervical biopsy specimens were fixed in 10% neutral buffered formalin

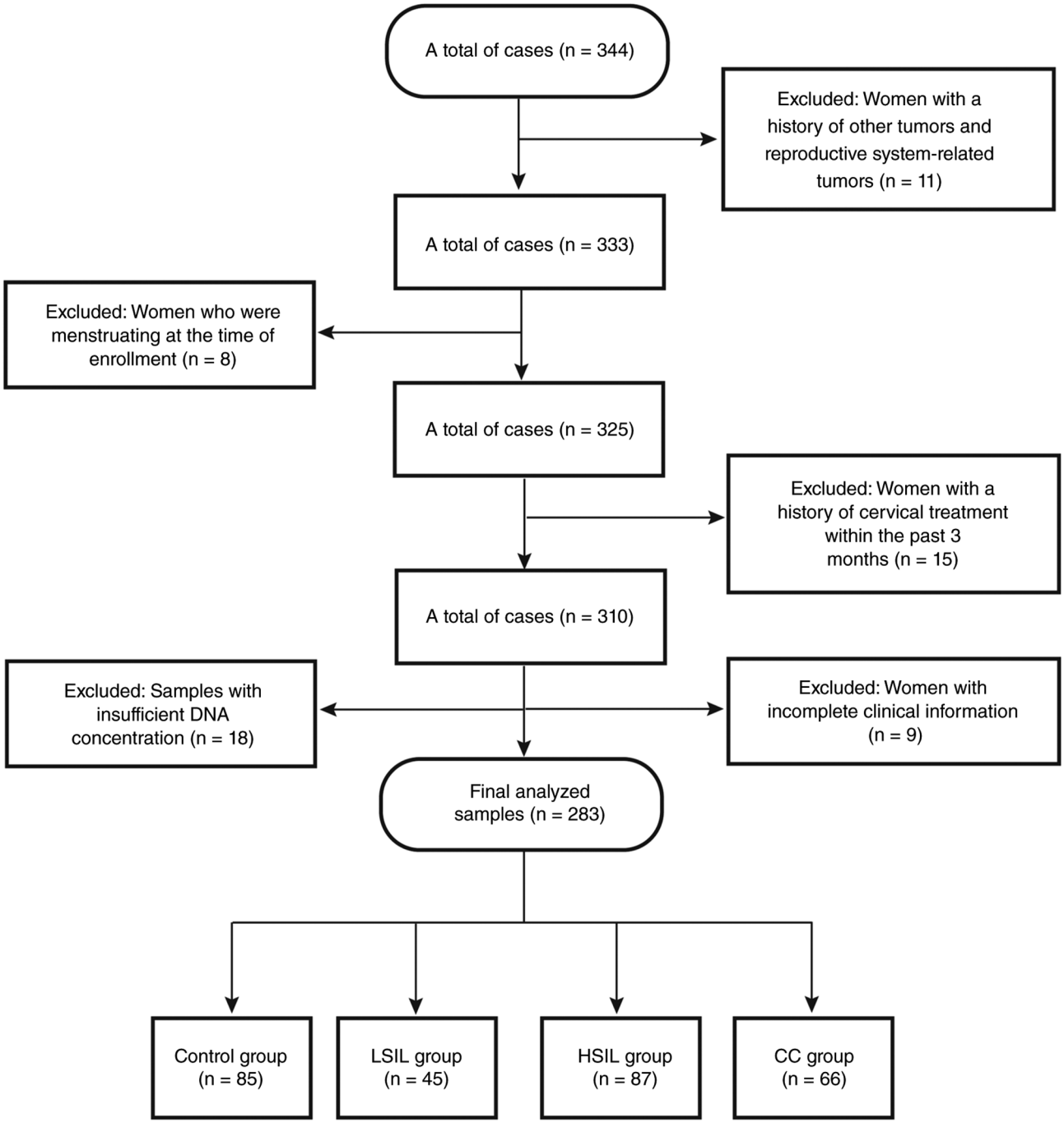

Figure 1. Flowchart of inclusion to the current analysis. The Control group refers to healthy participants without cervical lesions. LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical cancer.

at room temperature (~25˚C) for up to 24 h, embedded in paraffin and sectioned at 4 μm. The slides were stained with hematoxylin and eosin at room temperature (~25˚C) for 5 min in hematoxylin, followed by rinsing in running tap water for 5 min, differentiation in 1% acid alcohol for 3-5 sec, bluing in Scott's tap water substitute for 1 min and counterstaining with eosin for 2 min at room temperature. The slides were then independently reviewed by two senior pathologists with >8 years of experience in gynecological pathology using a light microscope (Olympus BX53; Olympus Corporation). Both pathologists were blinded to the results of methylation testing, HPV genotyping and liquid-based cytology. Discrepant diagnoses between the two pathologists were resolved by consensus. If consensus could not be reached, a third senior pathologist with >10 years of experience performed a blinded review to establish the final diagnosis. Inter-observer agreement between the two primary pathologists was assessed using Cohen's κ coefficient. The κ value was 0.86 (P<0.001), indicating excellent agreement.

Thin-prep cytology test (TCT) detection. According to the Bethesda System for reporting cervical cytology, LBC findings are classified as negative for intraepithelial lesion or

malignancy, atypical squamous cells of undetermined significance, atypical glandular cells of undetermined significance, LSIL, atypical squamous cells that cannot exclude HSIL, HSIL, squamous cell carcinoma and endocervical adenocarcinoma *in situ* (33).

DNA extraction, bisulfite treatment and methylation-specific PCR (MSP) analysis. Samples were prepared according to the manufacturer's instructions for the GynTect assay (Epitype GmbH), which includes the six markers ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671. The GynTect test kit received CE certification in October 2015, permitting its clinical use (34). DNA was extracted from frozen fresh cervical tissues using the GynTect Tissue Lysis Buffer Kit (Epitype GmbH) according to the manufacturer's instructions. Following tissue lysis, genomic DNA was purified and subjected to bisulfite conversion using the EpiTect® Fast Bisulfite Kit (Qiagen GmbH) according to the manufacturer's instructions. After bisulfite conversion and DNA elution, a 10 μl sample was added to each reaction mixture for the GynTect Real-Time Methylation-Specific PCR (qMSP) assay, which was performed using the ABI7500 Real-Time PCR System (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

The MSP protocol consisted of an initial denaturation at 94˚C for 60 sec, followed by 42 cycles of 94˚C for 15 sec and 67˚C for 30 sec. A melting curve analysis was subsequently performed (95˚C for 15 sec, 60˚C for 20 sec, 95˚C for 30 sec and 30˚C for 60 sec). Each PCR reaction was performed in a total volume of 20 μl containing 2.5 pmol of each methylation-specific primer and 5 ng of bisulfite-treated genomic DNA extracted from tissue sections. A sample was considered valid when each marker generated the corresponding melting curve after 42 cycles. Acetylcholinesterase (ACHE) was used as the internal reference for DNA quality control and the Cq value was required to be <36. Iduronate 2-sulfatase was used as the methylation quality control. All procedures were performed according to the manufacturer's recommended protocols.

After qMSP analysis, a Cq value was obtained for each marker. The ΔCq between each marker and the quality control marker ACHE was calculated as ΔCq=Cq(Marker)-Cq(ACHE). A ΔCq value of <9 for the five markers (ASTN1, DLX1, ITGA4, RXFP3 and SOX17) and ≤10 for ZNF671 was considered a positive result. When the ΔCq value of a marker met the corresponding positivity criterion, a predefined score was assigned. ASTN1, DLX1, ITGA4, RXFP3 and SOX17 were each assigned 1 point, whereas ZNF671 was assigned 3 points. A total score of ≥3 across the six markers was defined as a positive GynTect test result. The positivity criteria and scoring system were applied according to the GynTect manufacturer's protocol (Epitype GmbH). All cervical specimens were processed and stored according to standardized protocols before methylation testing. All laboratory personnel performing the assays were blinded to the histopathological diagnoses of the participants to minimize detection bias.

Statistical analysis. The primary analysis was defined as the comparison between the CIN2$^+$ group and the negative control group. Prior to the present study, the required sample size for primary diagnostic performance analysis was estimated *a priori* using the standard formula for diagnostic test evaluation:

$$n = \frac{Z^2_{1-\alpha/2} \times p(1-p)}{d^2}$$

In the above formula, p is the expected proportion, d is the margin of error, and $Z_{1-\alpha/2}$ is the critical value for a 95% confidence level (Z=1.96). Based on our preliminary data, the expected prevalence was estimated to be 0.85 (35). According to Daniel (36) (2012), the margin of error should be driven by the purposes of the study. For this exploratory diagnostic study, we therefore set the margin of error at 0.08 to balance statistical precision with practical feasibility. The calculation yielded a minimum sample size of ~77 participants. The present study exceeded this requirement and provided adequate statistical power for the primary analysis.

Notably, the diagnostic performance of individual genes was analyzed for exploratory purposes only. The primary clinical interpretation focused on the combined methylation panel and no formal correction for multiple comparisons was applied to the single-gene analyses.

Statistical analyses were performed using SPSS (version 26.0; IBM Corp.) and R (version 4.3.0; Posit Software, PBC). P<0.05 was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic performance of each methylation marker. The evaluated parameters included the area under the curve (AUC), sensitivity, specificity, positive predictive value, negative predictive value, Youden index (sensitivity + specificity-1) and their corresponding 95% CIs. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test, one-way ANOVA was used for comparisons among groups. For categorical variables, Fisher's exact test or the Pearson χ^2 test was used to assess differences between groups.

Results

Characteristics of study participants. A total of 283 women were included in the final analysis, comprising the control (30.0%), LSIL (15.9%), HSIL (30.7%) and CC (23.3%) groups. The age distribution in each group was as follows: control group, 27-77 years (mean, 51.07±11.49 years); LSIL group, 35-69 years (median, 54.00 years); HSIL group, 27-72 years (mean, 51.90±9.76 years) and CC group, 27-72 years (mean, 50.76±10.04 years). Except for the LSIL group, age was normally distributed in all groups and the test for homogeneity of variances indicated equal variances (P=0.806) (data not shown). Significant differences were observed among the groups in cytology results, HPV16/18 infection and GynTect test results (P<0.001). In addition, HPV16/18 infection and positive GynTect results were found to be more frequent in the HSIL and CC groups, which also exhibited higher methylation scores (Table I).

Three detection methods show diagnostic value in cervical lesions. For the diagnosis of HSIL and CC, the GynTect assay showed a sensitivity of 82.0 and a specificity of 87.0% (Table II). The sensitivity and specificity of HPV16/18 detection for HSIL and CC were 67.0 and 85.0%, respectively. The

Table I. Findings from cervical cancer screening and GynTect® tests in cases of precancerous CC.

Variable	Histological group				Total	P-value
	Control	LSIL	HSIL	CC		
Cytology results, n						<0.001[a]
NILM	73	10	18	14	115	
≥ASC-US	12	35	69	52	168	
HPV 16/18 results, n						<0.001[a]
Positive	2	18	53	50	123	
Negative	83	27	34	16	160	
GynTect assay, n						<0.001[a]
Positive	8	9	65	60	142	
Negative	77	36	22	6	141	
Score, median (Q25-Q75)	1.00 (0.00-1.00)	1.00 (0.00-2.00)	5.00 (2.50-7.00)	7.00 (6.00-8.00)	3.00 (0.00-7.00)	<0.001[a]

[a]P<0.001. GynTect, a diagnostic test of DNA methylation analysis of a methylation marker panel, including six markers (astrotactin 1, distal-less homeobox 1, integrin subunit α 4, relaxin family peptide receptor 3, SOX17 and ZNF671, zinc finger protein 671). LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical cancer; HPV, human papillomavirus; NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance.

Table II. Diagnostic performance of three assays for the diagnosis of high-grade squamous intraepithelial lesions and cervical cancer.

Detection method	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Youden index
HPV 16/18	0.76 (0.70-0.82)	0.67 (0.60-0.74)	0.85 (0.79-0.89)	0.52
TCT detection	0.72 (0.65-0.78)	0.79 (0.73-0.84)	0.64 (0.55-0.72)	0.43
DNA methylation tests targeting six genes	0.84 (0.79-0.89)	0.82 (0.76-0.86)	0.87(0.82-0.91)	0.69

AUC, area under the curve; HPV, human papillomavirus; TCT, thin-prep cytology test.

sensitivity and specificity of cytology for HSIL and CC were 79.0 and 64.0%, respectively. The GynTect assay demonstrated higher specificity compared with HPV16/18 detection and cytology. In addition, the six-gene DNA methylation assay achieved the highest AUC (0.84) and the highest Youden index (0.69; Fig. 2). In comparison, HPV16/18 detection yielded an AUC of 0.76 with a Youden index of 0.52, whereas cytology achieved an AUC of 0.77 with a Youden index of 0.43 (Table II).

Decision curve analysis (DCA) was performed in the present study. Unlike conventional diagnostic indicators, such as sensitivity and specificity, DCA comprehensively evaluates the net clinical benefit of different screening strategies across a range of threshold probabilities. The present DCA results demonstrated that the methylation panel provided a higher net clinical benefit over a broader range of threshold probabilities compared with HPV detection (Fig. 3) and TCT (Fig. 4) alone. Within the optimal threshold range for clinical decision-making, the methylation-based model demonstrated a superior predictive performance and greater clinical utility compared with the conventional screening methods (Fig. 5).

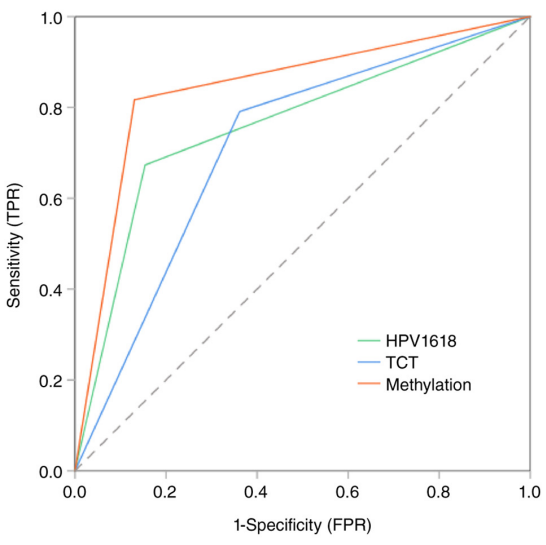

Figure 2. Receiver operating characteristic curves of three assays for the diagnosis of high-grade squamous intraepithelial lesions and cervical cancer. TPR, true positive rate; FPR, false positive rate; HPV, human papillomavirus; TCT, thin-prep cytology test.

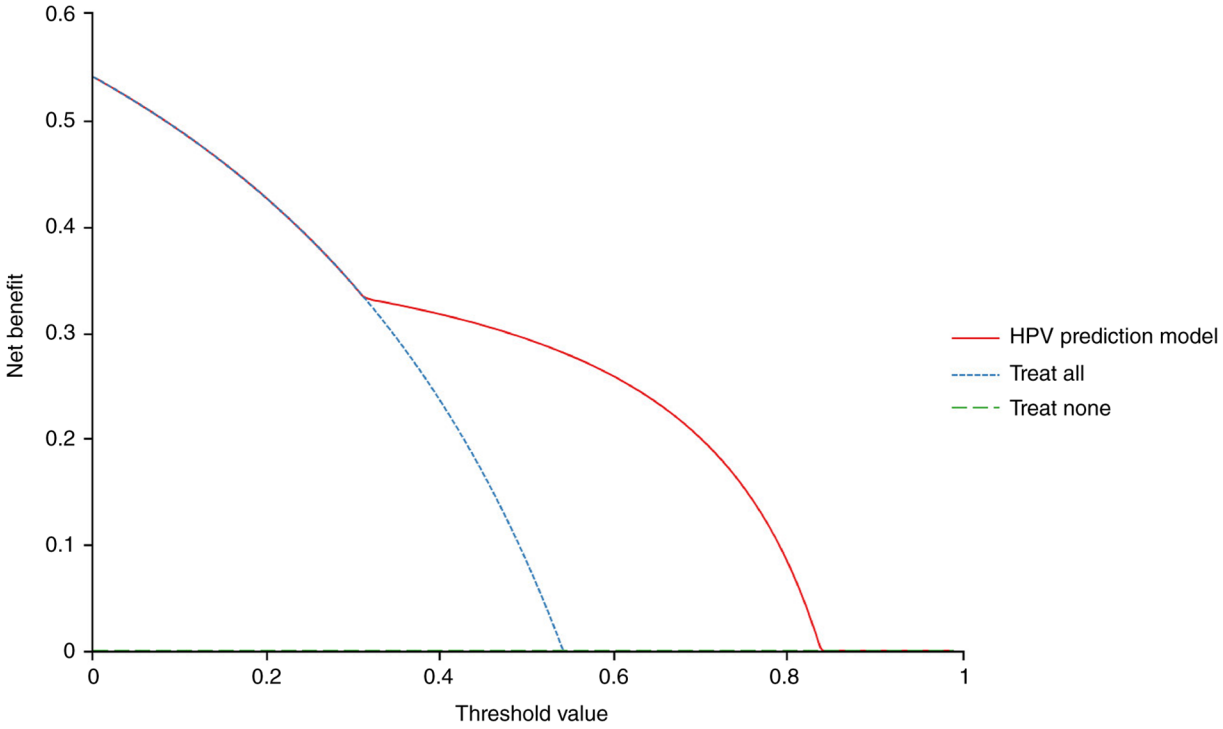

Figure 3. Decision curve analysis of the HPV prediction model. HPV, human papillomavirus.

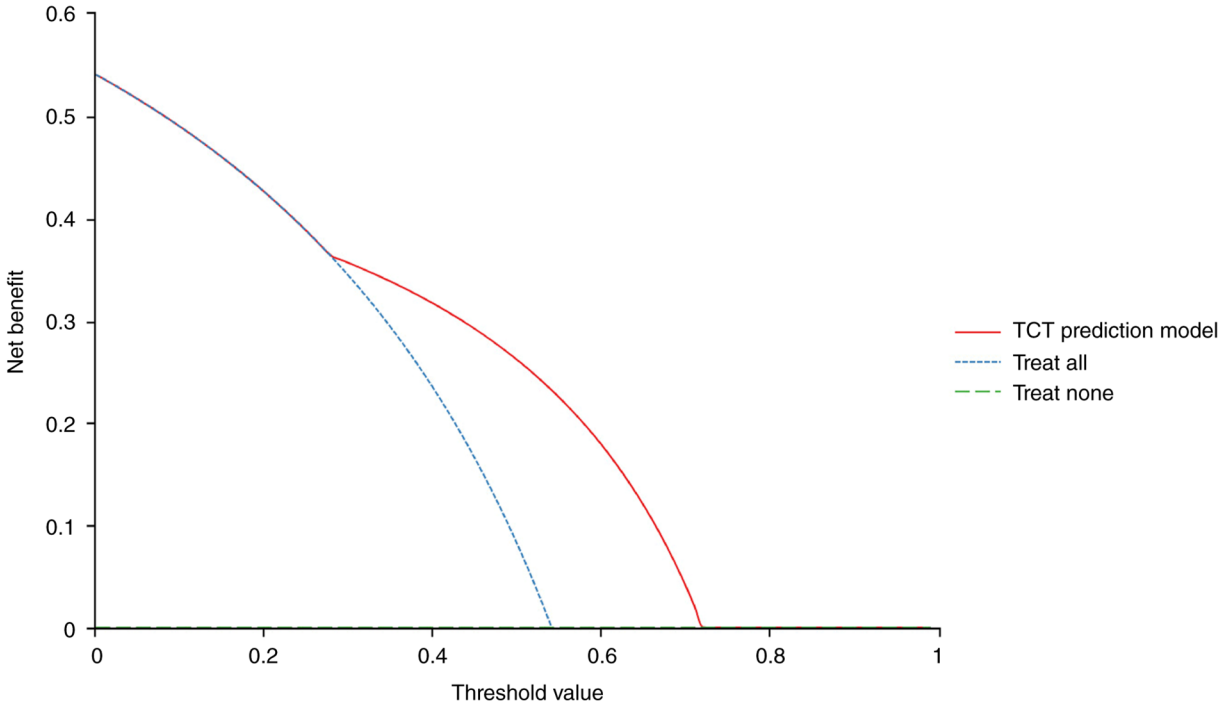

Figure 4. Decision curve analysis of the TCT prediction model. TCT, thin-prep cytology test.

As shown in Table III, the methylation panel demonstrated significantly improved reclassification and discrimination performance compared with HPV and TCT (all, P<0.001). The overall NRI was 1.37 for both comparisons. The IDI was 0.20 compared with HPV and 0.28 compared with TCT. These findings indicate that the methylation model exhibited a superior predictive performance for cervical lesion screening.

Analysis of methylation positive rates in HSIL and CC groups. In the CC group, the positive methylation rates of ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671 were 84.85, 87.88, 69.70, 75.76, 68.18 and 89.39%, respectively, with ZNF671 showing the highest positive methylation rate. In the HSIL group, ITGA4 exhibited the lowest positive methylation rate (34.48%), whereas DLX1 had the highest positive methylation

Table III. NRI and IDI of the methylation panel compared with HPV and TCT models.

Comparison	Overall NRI (95% CI)	NRI^+ (95% CI)	NRI^- (95% CI)	IDI (95% CI)	P-value
Methylation vs. HPV	1.37 (1.20-1.54)	0.63 (0.51-0.76)	0.74 (0.62-0.85)	0.20 (0.13-0.26)	<0.001
Methylation vs. TCT	1.37 (1.20-1.54)	0.63 (0.51-0.76)	0.74 (0.62-0.85)	0.28 (0.21-0.35)	<0.001

NRI, net reclassification improvement; NRI^+, NRI for cases (high-grade squamous intraepithelial lesions/cervical cancer; NRI^-, NRI forcontrols (Control/low-grade squamous intraepithelial lesions); IDI, integrated discrimination improvement; HPV, human papillomavirus; TCT, thin-prep cytology test.

Table IV. Genes with positive DNA methylation rates in various cervical lesion groups.

	LSIL group		HSIL group		CC group	
Gene	Positive, n	Rate, %	Positive, n	Rate, %	Positive, n	Rate, %
ASTN1	14	31.11	57	65.52	56	84.85
DLX1	29	64.44	65	74.71	58	87.88
ITGA4	1	2.22	30	34.48	46	69.70
RXFP3	6	13.33	31	35.63	50	75.76
SOX17	8	17.78	38	43.68	45	68.18
ZNF671	1	2.22	61	70.11	59	89.39

ASTN1, astrotactin 1; DLX1, distal-less homeobox 1; ITGA4, integrin subunit α 4; RXFP3, relaxin family peptide receptor 3; ZNF671, zinc finger protein 671.

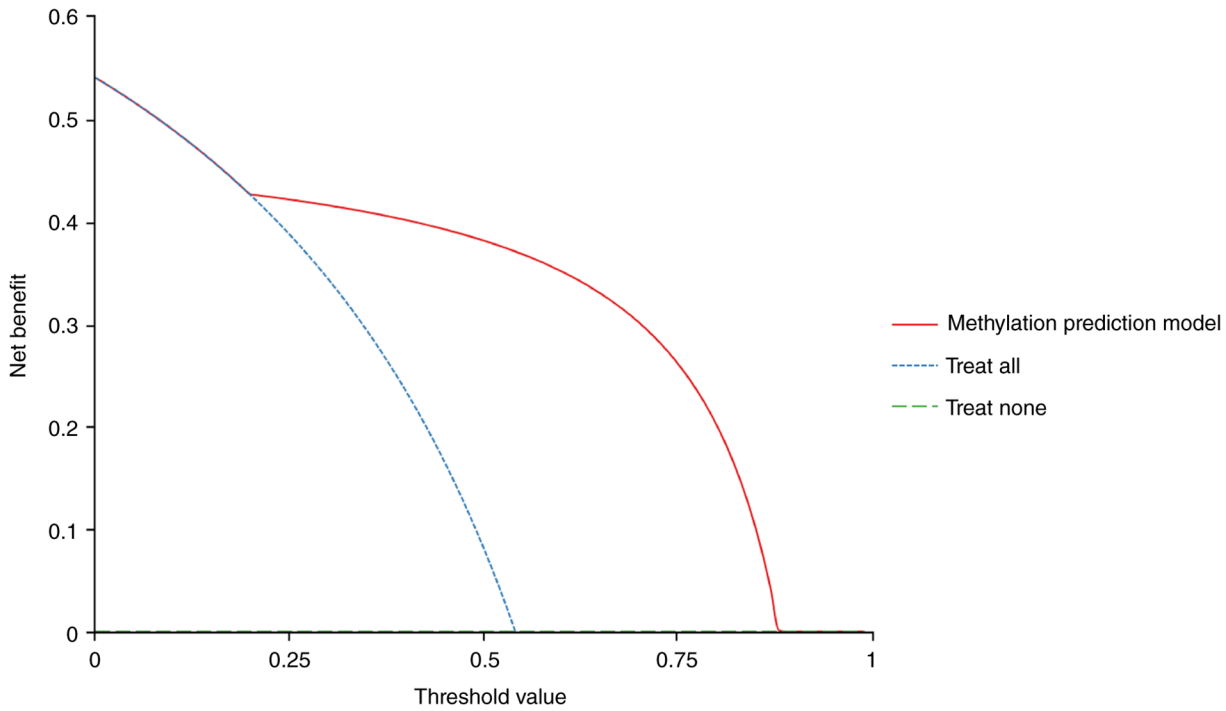

Figure 5. Decision curve analysis of the methylation prediction model.

rate (74.71%; Table IV). In addition, Fig. 6 illustrates the proportions of positive and negative methylation results for the six markers across the different groups. DNA methylation scores differed significantly among the Control, LSIL, HSIL and CC groups (Fig. 7). Comparisons show that the methylation score was significantly lower in the Control group than that in the LSIL group ($P<0.05$). Furthermore, the CC group had significantly higher methylation scores than the Control,

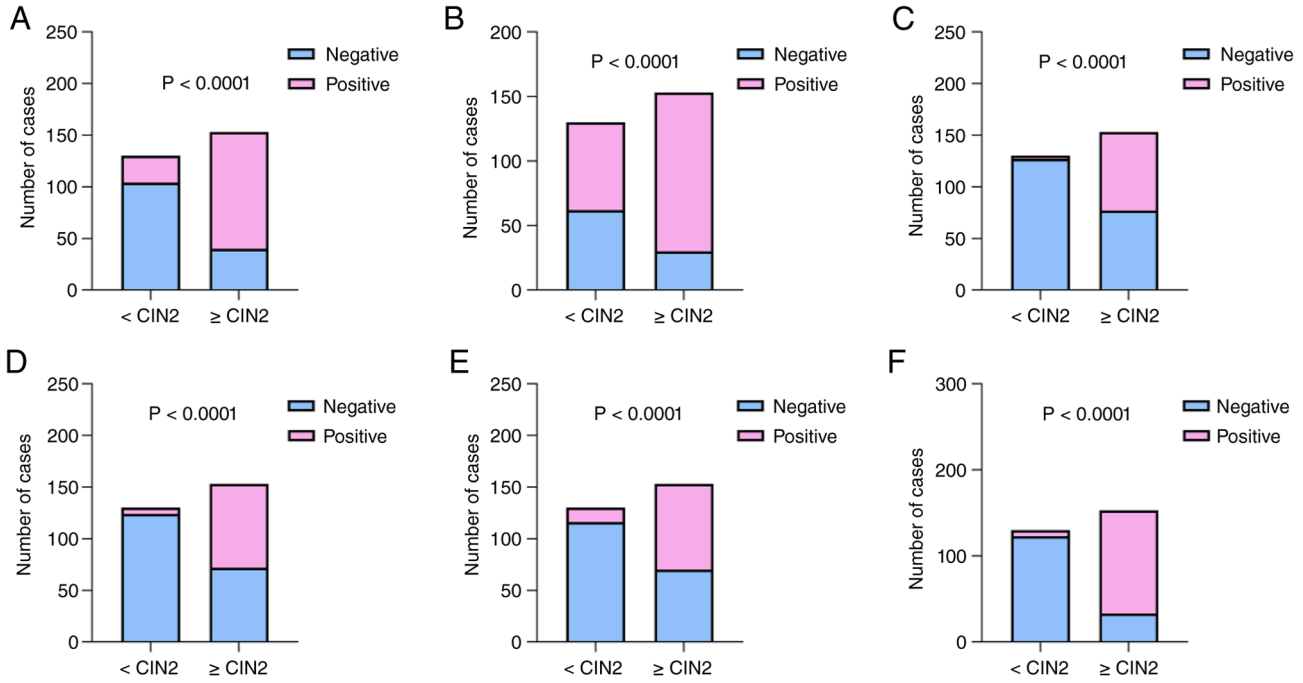

Figure 6. Methylation percentage of six genes within various groups. (A) astrotactin 1, (B) distal-less homeobox 1, (C) integrin subunit α 4, (D) relaxin family peptide receptor 3, (E) SOX17 and (F) zinc finger protein 671. CIN2, cervical intraepithelial neoplasia grade 2.

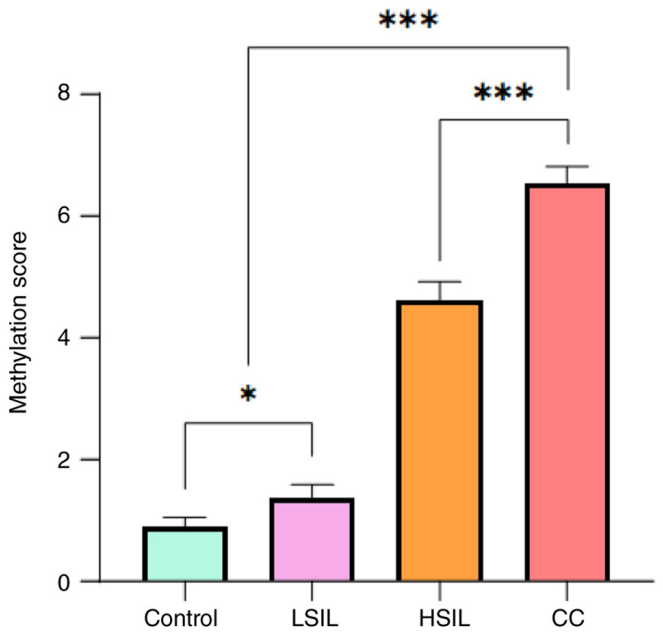

Figure 7. Methylation score in cervical precancerous and cancerous lesions. Data represent the mean ± SEM. *P<0.05 and ***P<0.001. LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical cancer.

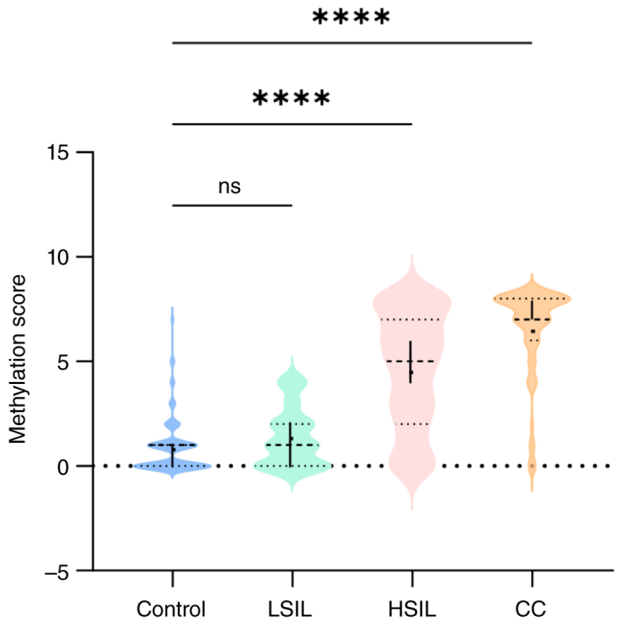

Figure 8. Methylation scores across four histological groups. Individual data are displayed through violin plots and jitter points. ****P<0.0001. LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical cancer; ns, not significant.

LSIL and HSIL groups (all P<0.001). In addition, violin plots with jittered data points illustrate the distribution of individual methylation scores. The mean methylation scores were 0.91 for the control group, 1.38 for the LSIL group, 4.64 for the HSIL group and 6.55 for the CC group. The corresponding 95% CIs for the medians were 0.00-1.00, 0.00-2.00, 4.00-6.00 and 7.00-8.00, respectively. Both indicators showed a gradual increase with disease progression. Notable variability within each group was also observed. Comparisons showed no significant difference between the control and LSIL groups, whereas the HSIL and CC groups exhibited significantly higher methylation levels (P<0.0001; Fig. 8).

Six-gene methylation tests demonstrate good diagnostic performance for detecting HSIL and CC. As shown in Fig. 9, ROC curves and AUC analyses were used to evaluate the diagnostic

Table V. Diagnostic performance of DNA methylation tests targeting six genes for the diagnosis of high-grade squamous intraepithelial lesions and cervical cancer.

Gene	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Youden index
ASTN1	0.77 (0.71-0.83)	0.74 (0.67-0.80)	0.80 (0.73-0.85)	0.81(0.74-0.87)	0.72 (0.64-0.79)	0.54
DLX1	0.64 (0.58-0.71)	0.80 (0.75-0.85)	0.48 (0.39-0.56)	0.64 (0.57-0.71)	0.67 (0.57-0.77)	0.28
ITGA4	0.74 (0.68-0.80)	0.50 (0.42-0.58)	0.98 (0.93-0.99)	0.96 (0.90-0.99)	0.62 (0.55-0.69)	0.48
RXFP3	0.74 (0.68-0.80)	0.53 (0.45-0.61)	0.95 (0.90-0.98)	0.93 (0.86-0.97)	0.63 (0.56-0.70)	0.48
SOX17	0.72 (0.66-0.78)	0.54 (0.46-0.62)	0.89 (0.84-0.93)	0.86 (0.77-0.91)	0.62 (0.55-0.69)	0.43
ZNF671	0.87 (0.82-0.91)	0.78 (0.72-0.83)	0.95 (0.89-0.98)	0.94 (0.89-0.97)	0.79 (0.72-0.85)	0.73
Combined	0.84 (0.79-0.89)	0.82 (0.76-0.86)	0.87 (0.82-0.91)	0.88 (0.81-0.93)	0.80 (0.72-0.86)	0.69

ASTN1, astrotactin 1, DLX1, distal-less homeobox 1; ITGA4, integrin subunit α 4; RXFP3, relaxin family peptide receptor 3; ZNF671, zinc finger protein 671; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

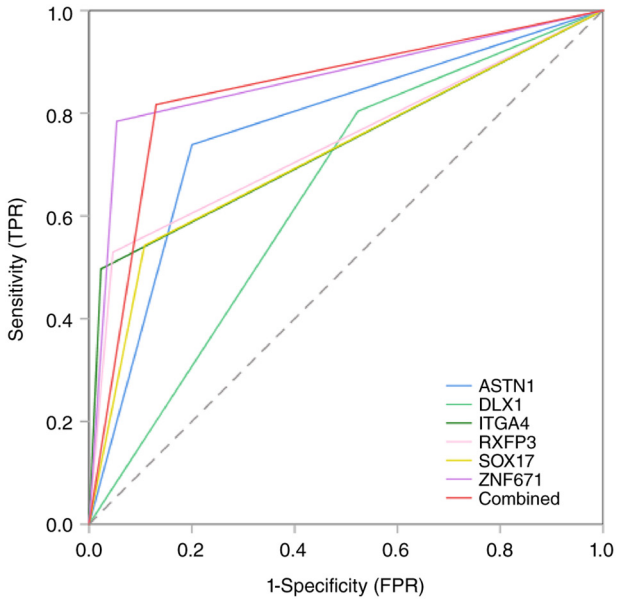

Figure 9. Receiver operating characteristic curves of six genes methylation tests for the diagnosis of high-grade squamous intraepithelial lesions and cervical cancer. TPR, true positive rate; FPR, false positive rate; ASTN1, astrotactin 1; DLX1, distal-less homeobox 1; ITGA4, integrin subunit α 4; RXFP3, relaxin family peptide receptor 3; ZNF671, zinc finger protein 671.

performance of the individual methylation biomarkers. All six methylation markers achieved AUC values >0.50. As shown in Table V, among the individual markers, ZNF671 had the highest AUC (0.87). DLX1 demonstrated the highest sensitivity (80.0%) but the lowest specificity. Among the six genes, ITGA4 showed the highest specificity (98.0%) but a sensitivity of only 50.0%. Among the single-gene methylation assays, ZNF671 exhibited the best diagnostic performance, with a sensitivity of 78.0%, a specificity of 95.0% and a Youden index of 0.73. Although the six-gene combined assay yielded a lower AUC than the ZNF671 assay alone, it achieved higher sensitivity and could effectively reduce the risk of missed diagnosis. These Youden index values indicate that the combined methylation assay provides a favorable balance between sensitivity and specificity for detecting HSIL and CC.

Discussion

Screening based on high-risk HPV testing and cytology has been reported to have limited sensitivity for detecting HSIL and CC (37,38). In addition, the relatively low specificity exhibited may lead to an increased number of unnecessary colposcopy referrals, thereby imposing additional physical and psychological burdens on patients. The present findings showed that the positive methylation rates of the six-gene panel increased progressively from the control group to the LSIL, HSIL and CC groups, reaching 9.41, 20.0, 74.7 and 90.9%, respectively. The positive methylation detection rate increased with the severity of cervical lesions. Compared with single-gene detection, the combined six-gene assay demonstrated higher sensitivity (82.0%) for the diagnosis of HSIL and more advanced lesions, with an AUC of 0.84 (95% CI: 0.79-0.89) and a Youden index of 0.69. The single-gene ZNF671 model exhibited excellent overall diagnostic performance and may be an appropriate option for general population screening. By contrast, the six-gene combined model provided higher sensitivity, thereby reducing the risk of missed diagnosis and offering valuable support for the auxiliary diagnosis of high-risk populations, borderline specimens and suspected cases.

A previous study reported that the methylation-positive group had a markedly higher risk of cervical intraepithelial neoplasia grade 2 or worse ($CIN2^+$) compared with the methylation-negative group, with an odds ratio of 11.3. Methylation-based screening reduced the colposcopy referral rate by 67.2% while achieving a sensitivity of 83.0% and a specificity of 69.9% for the detection of $CIN2^+$ (39). A recent study also demonstrated that combined testing of the same six loci achieved higher specificity compared with high-risk HPV testing for the detection of $CIN2^+$/$CIN3^+$ (25). These findings further support the favorable diagnostic performance of the combined methylation assay for cervical precancerous lesions.

The present study also demonstrated that the combined detection of the six genes showed superior diagnostic performance for HSIL and CC. Compared with HPV16/18 testing and TCT, the six-gene combined assay achieved higher AUC and sensitivity for the detection of HSIL and CC. These findings indicate that DNA methylation detection exhibits a

superior diagnostic performance compared with HPV16/18 testing and TCT and can effectively distinguish different grades of cervical lesions. Compared with conventional HPV- and TCT-based models, the methylation panel demonstrated greater net clinical benefit in the DCA. This finding suggests that the methylation panel may reduce unnecessary referrals among low-risk individuals while maintaining reliable triage performance for high-risk populations, thereby supporting its potential clinical value and cost-effectiveness in CC screening. Furthermore, the marked improvements in NRI and IDI indicate that the methylation panel provides meaningful clinical advantages compared with conventional screening methods. The high NRI values suggest that the model more accurately classifies patients into appropriate risk categories, reducing both unnecessary referrals among low-risk individuals and missed identification of high-risk cases. The notable improvement in IDI further demonstrates its superior ability to discriminate between patients with and without cervical lesions, supporting its potential as a more effective triage tool for CC screening.

However, previous research regarding methylation detection has primarily focused on the diagnostic performance of combined methylation panels (40,41). In the present study, in addition to evaluating the diagnostic performance of the six-gene panel, the diagnostic value of each individual gene was also assessed. Previous studies have shown that, compared with HPV16/18 genotyping and PAX1 methylation detection, ZNF671 methylation has superior diagnostic performance for $CIN2^+$, particularly in women who are hrHPV-negative but have $CIN2^+$ (16). An additional study identified ZNF671 as an important tumor suppressor in colorectal carcinoma (CRC) and reported that reduced ZNF671 expression was associated with poor patient survival (42). Hypermethylation of the ZNF671 promoter was observed in CRC tissues and was inversely associated with ZNF671 expression. Treatment with demethylating agents restored ZNF671 expression in CRC cell lines and upregulation of ZNF671 suppressed the proliferative and metastatic capacities of CRC cells. In addition, research has reported that overexpression of ZNF671 inhibits the proliferation and metastasis of non-small cell lung cancer cells by suppressing the Wnt/β-catenin signaling pathway (43). Collectively, these findings indicate that ZNF671 exhibits tumor-suppressive properties across a number of malignancies. In the present study, the positive methylation rates of ZNF671 were 2.22, 70.11 and 89.39% in the LSIL, HSIL and CC groups, respectively. Among the single-gene methylation assays, ZNF671 demonstrated the best diagnostic performance, with an AUC of 0.87 (95% CI: 0.82-0.91), as well as high sensitivity and specificity for detecting HSIL and CC.

A number of limitations within the present study should also be acknowledged. First, LSIL is generally a self-limiting lesion with a high rate of spontaneous regression. Numerous affected individuals undergo only screening and follow-up at primary healthcare institutions and were therefore not included in the present study, resulting in a relatively small sample size for the present subgroup. Second, given that this was a single-center retrospective study using convenience sampling, potential selection bias cannot be completely excluded, which may limit the generalizability of the present findings. In addition, cut-off determination and diagnostic performance evaluation were both conducted using the same internal dataset without independent external validation, which may have introduced optimism bias and increased the risk of overfitting. Furthermore, as this was a retrospective study, FAM19A4/miR124-2 methylation, a triage biomarker widely recommended in international CC screening guidelines, was not evaluated simultaneously (44). Consequently, the present study was unable to directly compare the diagnostic performance of the present panel with that of this biomarker. Future large-scale, multicenter prospective studies incorporating parallel evaluation of both methylation panels are warranted to further assess their clinical value, triage performance and cost-effectiveness, thereby identifying the optimal methylation panel for CC screening in the Chinese population.

In conclusion, the present study demonstrated that detection of DNA methylation in six genes (ASTN1, DLX1, ITGA4, RXFP3, SOX17 and ZNF671) in cervical cells showed good diagnostic performance for CC and HSIL. Among the three detection methods evaluated in the present study, the combined six-gene methylation assay achieved the highest sensitivity. This approach may have potential clinical value in the diagnosis of CC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HG, XC and JL conceptualized the present study. HG and JL curated the data. HG and JL acquired the funding. LT, SM, JG and LH were responsible for the methodology, including experimental design and protocol development. HG provided supervision. XC and JL wrote the original manuscript draft. XL and XY contributed to clinical data acquisition and interpretation. XC and JL confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of General Hospital of Ningxia Medical University (Yinchuan, China; approval no. KYLL-2024-1471).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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