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Analysis of the triage value of multigene methylation testing for CIN2 + in hrHPV-positive patients

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Abstract

Objective To assess the triage value of multigene methylation testing for cervical intraepithelial neoplasia 2 and above (CIN2+) in high-risk human papillomavirus (hrHPV) positive patients.

Methods 634 hrHPV-positive cases were selected from the gynecology outpatient clinic at Hainan Women and Children's Medical Center between July 2022 and April 2024. Out of these, 274 patients were excluded based on the inclusion and exclusion criteria. A total of 360 patients were evaluated for hrHPV, cytology, histopathology, and DNA methylation across multiple loci. These patients were categorized into five groups based on their histopathological diagnoses: control group, CIN1 group, CIN2 group, CIN3 group, and cervical cancer (CC) group. The triage value of multigene methylation testing for CIN2 + in hrHPV-positive patients was evaluated by calculating the positivity of candidate gene methylation, sensitivity, specificity, area under the curve (AUC), and other performance indicators.

Results Among the 17 candidate genes (ST6GALNAC5, PAX1, AJAP1, CDKN2A, ZNF671, GATA4, MAL, POU4F3, RXFP3, JAM3, MIR124, LHX8, SOX1, ASTN1, SOX17, DLX1, and ITGA4), ITGA4 methylation testing demonstrated the highest diagnostic efficacy for detecting CIN2 + lesions, with an AUC of 0.866 (95% confidence interval [CI]: 0.806–0.925). This method exhibited a sensitivity of 75.32% (95% CI: 0.647–0.836) and a specificity of 96.45% (95% CI: 0.936–0.981). The combined methylation test, which included all candidate genes, showed a higher specificity of 97.87% (95% CI: 0.954–0.990) compared to any individual gene methylation test. However, its sensitivity was lower, at 72.73% (95% CI: 0.619–0.814). Furthermore, the diagnostic accuracy of combining HPV16/18 testing with all candidate gene methylation tests for the diagnosis of CIN2 + was significantly greater than when HPV16/18 testing was combined with cytology. This combined approach had an AUC of 0.907 (95% CI: 0.858–0.955), a sensitivity of 72.73% (95% CI: 0.619–0.814), and a specificity of 98.58% (95% CI: 0.964–0.995).

Conclusion Multigene methylation testing is an efficient triage test for CIN2 + in hrHPV-positive patients and has potential value in clinical practice. Combined HPV16/18 and multigene methylation testing for the triage of CIN2 + is significantly better than combined HPV16/18 and cytology testing.

Keywords Cervical cancer, Cervical intraepithelial neoplasia, Multigene methylation testing, Triage value

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Introduction

Since 2000, the incidence of cervical cancer (CC) in China has increased significantly, and the latest statistics show that CC ranks as the fourth most commonly diagnosed cancer and the most common gynecological cancer among women in the world, with 604,127 newly diagnosed cases and 341,831 deaths [1, 2]. CC progresses through three grades of premalignant lesions known as cervical intraepithelial neoplasia (CIN) 1 to 3 (CIN1–3) [3]. CIN2 and CIN3 are considered high-risk lesions and are typically treated with resection or ablation, although they may sometimes regress independently [4]. The standard treatment for both CIN2 and CIN3 involves excising the transformation zone of the cervix, which is highly effective. However, even after treatment, there remains an elevated risk of developing cervical cancer for up to 20 years [5]. According to the latest U.S. guidelines published in 2020 [6], treating all cases of CIN2 and above (CIN2+) is recommended. However, treatment may be deferred if the risk of complications during future pregnancies is greater than the risk of developing cancer [6]. Persistent infection with a high-risk human papillomavirus (hrHPV) is essential in developing CC and its precursors, but it can take decades for hrHPV infection to progress to CC [7, 8]. hrHPV vaccination and screening have reduced both morbidity and mortality from CC in many high-income countries, but incidence remains high in resource-limited low- and middle-income countries [7, 8]. Today, cytology and/or HPV-based screening are the most widely used CC screening methods worldwide [9]. However, the sensitivity and specificity of cytologic methods are affected by subjective factors such as sampling techniques and the interpretation skills of the pathologist [10]. HPV testing also detects transient, clinically irrelevant HPV infections, resulting in lower specificity than cytology [11]. All of the above may lead to an increase in colposcopy referral rates, thereby increasing the physical and psychological burden on women, making it particularly important to develop effective triage methods to predict the occurrence of high-grade cervical lesions.

Aberrant DNA methylation has been shown to cause oncogene activation, oncogene silencing and inactivation, cell transformation, and imprint loss, leading to genetic instability and cancer [12]. DNA methylation is associated with the severity of CIN lesions and the risk of developing invasive cancer, and genes currently found to be hypermethylated in cervical cancer include PAX1, SEPT9, ASTN1, DLX1, ITGA4, RXFP3, SOX17, ZNF671, ZNF582, FAM19A4, CADM1, JAM3, MIR124, MAL, ST6GALNAC5, CDKN2A, LHX8, POU4F3, GATA4, AJAP1, ASCL1 and SOX1, among others [7, 10, 13–19]. Among them, a study in China concluded that the methylation levels of PAX1 and SEPT9 increased with the severity of cervical lesions and could be effective and powerful biomarkers for the diagnosis of CC/pre-cancerous lesions and could be used as an alternative triage test in HPV-based CC/pre-cancerous lesions screening programs [7]. Shi et al. [20] found that the diagnostic value of methylated biomarkers for CC/pre-cancerous lesions demonstrated excellent diagnostic efficacy when screening samples with CIN2 + using a six-gene kit (ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671). Zhang et al. [21] found that the use of methylation screening reduced colposcopy referrals by 67.2%, with sensitivity and specificity of 83.0% and 69.9%, respectively, for the detection of CIN2+. Based on the above, host-cell DNA methylation testing is expected to be a biomarker for hrHPV-positive triage. However, the clinical manifestations of DNA methylation have varied in studies in different populations, and most studies based on DNA methylation biomarkers have been conducted by different research groups in different populations using various analytical methods. Therefore, evaluating potential candidate genes for cervical lesion triage in the same population is necessary to provide an additional reference for clinical triage of hrHPV-positive patients. In this study, we examined 17 candidate genes: ST6GALNAC5, PAX1, AJAP1, CDKN2A, ZNF671, GATA4, MAL, POU4F3, RXFP3, JAM3, MIR124, LHX8, SOX1, ASTN1, SOX17, DLX1, and ITGA4. These genes were selected based on previously reported findings related to methylation detection. We detected the methylation of these genes in cervical squamous cell samples that are positive for hrHPV using high-throughput sequencing. We identified the top candidate gene for methylation analysis from a selection of 17 genes and assessed the usefulness of multigene methylation testing for triaging women with hrHPV who are CIN2 + positive.

Methods

Study population

Participant recruitment

This study received approval from the Medical Ethics Committee of Hainan Women and Children's Medical Center (ethics number: HNWCMC MEC No. 095 of 2022), China, and was conducted in accordance with the Declaration of Helsinki. The inclusion criteria for all subjects were as follows: (1) age between 18 and 70 years; (2) no history of uterine surgery; (3) no use of vaginal medication or vaginal irrigation within 3 days prior to the visit; (4) the patient must agree to participate in the study and sign an informed consent form. The exclusion criteria for all subjects were as follows: (1) individuals without cervical lesions detected by colposcopy; (2) those with incomplete clinical information, insufficient DNA concentration in the sample, or missing samples; (3) individuals who are pregnant, breastfeeding, or have a history

of tumors in the reproductive tract; (4) individuals who are menstruating or within two days of their menstrual period at the time of enrollment. Between July 2022 and April 2024, we recruited 634 patients who tested positive for hrHPV during routine screening at Hainan Women and Children's Medical Center's gynecology outpatient clinic and exhibited no associated clinical symptoms. After applying the inclusion and exclusion criteria, 274 samples were excluded, leaving 360 eligible samples for statistical analysis (as shown in Fig. 1). We collected the subjects' clinic numbers, ages, hrHPV results, cytology results, colposcopy findings, and cervical tissue biopsy results.

Detection of HrHPV

The hrHPV test kit (Hybrebio, Chaozhou) is used for the qualitative detection of 14 hrHPV DNA types, specifically types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. This assay is validated for its effectiveness. The hrHPV genotypes are divided into two categories: those that are positive for types 16 and/or 18 and those that are hrHPV-positive but do not include types 16 or 18. A genotype is classified as 16/18-positive if it tests positive for both types 16/18 and any other hrHPV types.

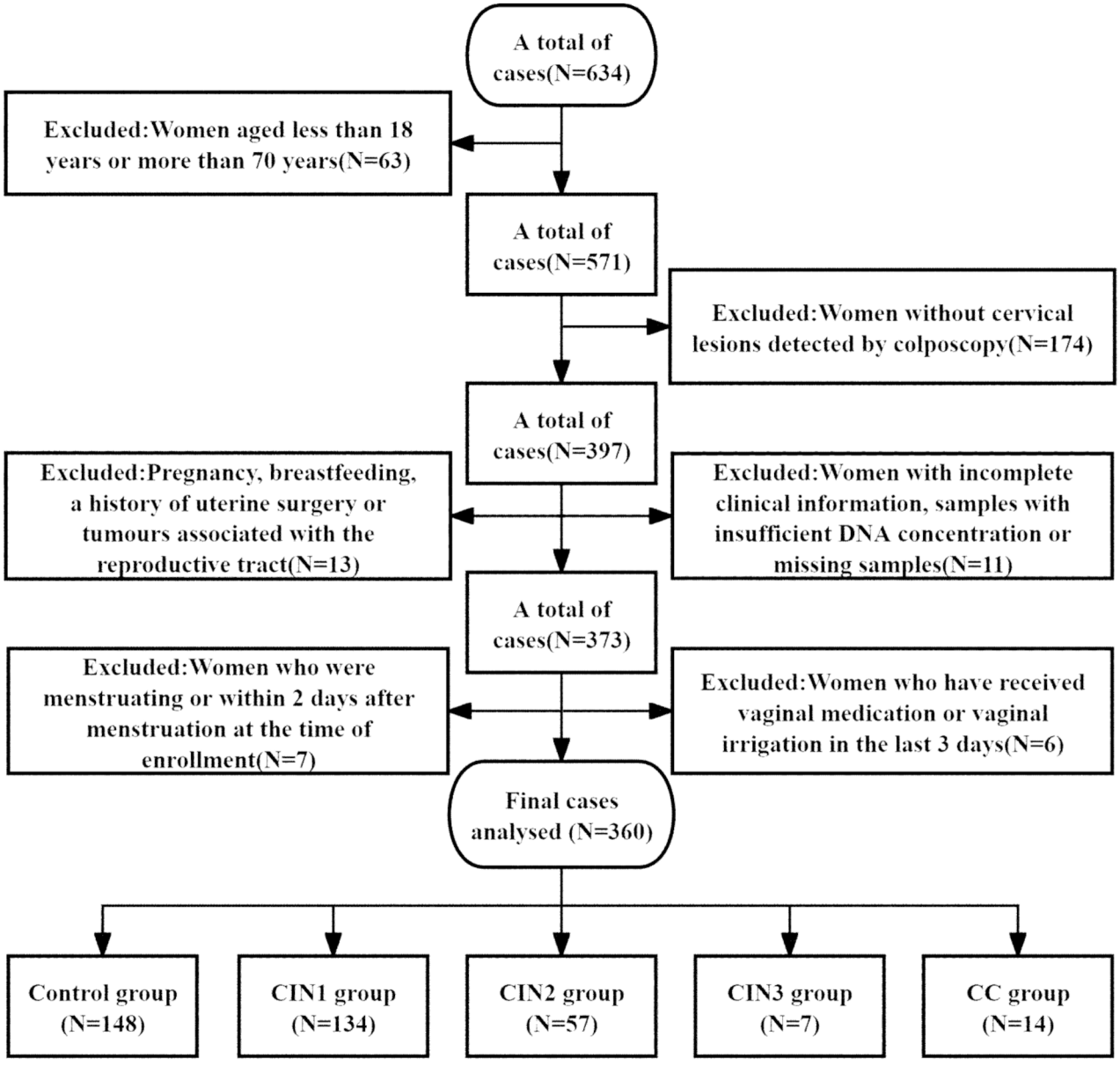

Fig. 1 Flowchart of inclusion criteria for the current analysis

Cytology testing

Cytology testing was conducted using the ThinPrep Cytologic Test (TCT) method. According to the classification established by Perkins et al. in 2020, the cytology results were categorized based on the International Cancer Society's Bethesda System (TBS) for cervical cytology, which includes negative for intraepithelial lesion or malignancy (NILM), low-grade squamous intraepithelial lesion (LSIL), atypical squamous intraepithelial lesion of undetermined significance (ASCUS), atypical glandular cells of undetermined significance (AGC), high-grade squamous intraepithelial lesion (HSIL), atypical squamous cells that cannot exclude HSIL (ASC-H) squamous cell carcinoma (SCC), and endocervical adenocarcinoma in situ (AIS) [6].

Histopathological grouping criteria

Two experienced pathologists graded cervical histopathologic findings into five groups according to standard cervical lesion staging guidelines: control group, CIN1 group, CIN2 group, CIN3 group, and CC group. Sampling for candidate gene methylation detection was performed according to the sample collection procedure for cytology testing, with all samples collected and numbered by a fixed number of physicians during the examination and de-linked from clinical information prior to data analysis.

Host-cell gene methylation detection and information analysis

Cervical smear samples were collected using TCT sampling and stored in a TCT cell preservation solution at -20 °C. These samples were sent to Beijing Mackinaw Gene Technology Co., Ltd. for high-throughput methylation sequencing analysis of the following genes: ST6GALNAC5, PAX1, AJAP1, CDKN2A, ZNF671, GATA4, MAL, POU4F3, RXFP3, JAM3, MIR124, LHX8, SOX1, ASTN1, SOX17, DLX1, and ITGA4. The detailed process of the host-cell gene methylation detection and information analysis is shown in Fig. 2.

Experimental method for Host-cell gene methylation detection

The company initiated the process by randomly cleaving the genomic DNA to obtain short DNA fragments from a quality-controlled DNA sample. This was followed by end repair, annealing, and methylation crosslinking. After screening to select fragments of appropriate length, the DNA underwent bisulfite treatment and was amplified by PCR to create sequencing libraries. These libraries were sequenced on the MGISEQ-2000 sequencer after passing quality control checks to ensure they met the required standards.

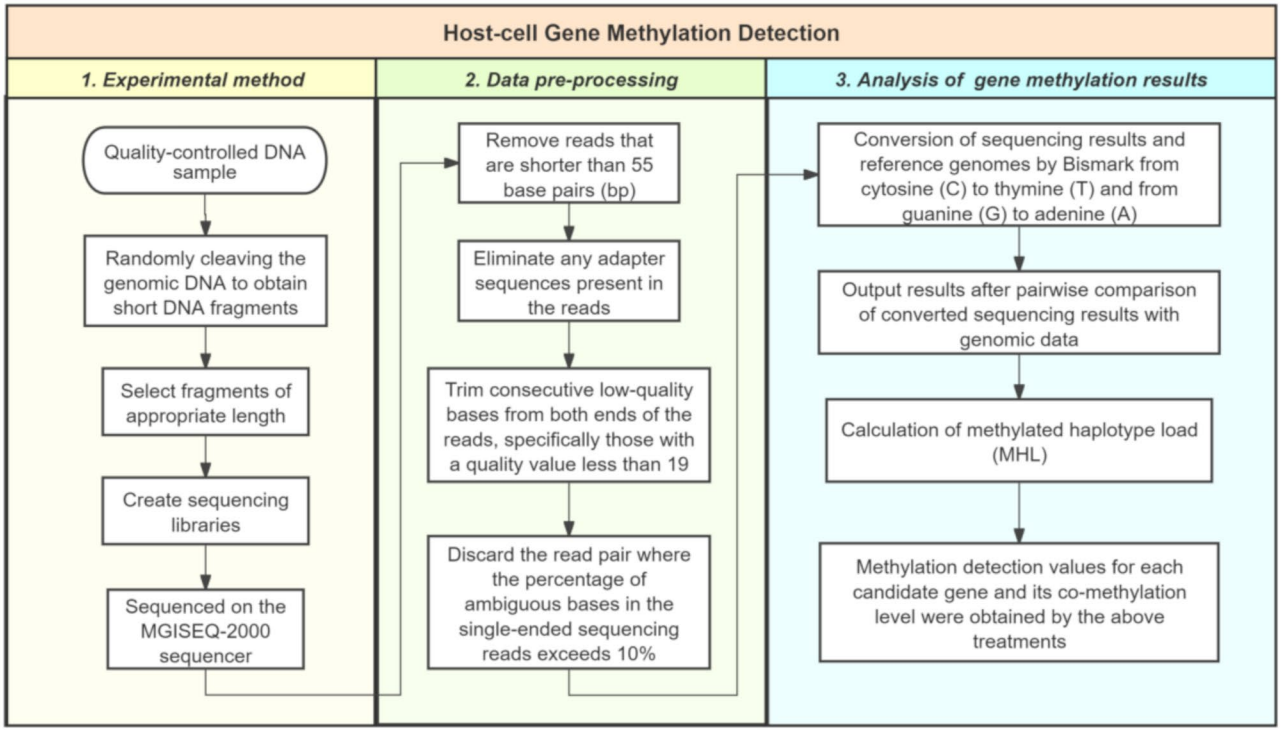

Fig. 2 Flowchart of the host-cell gene methylation detection and information analysis

Data Pre-processing

The raw reads obtained from sequencing often include fragmented and low-quality sequences, complicating subsequent analyses. To ensure the reliability of the data analysis, these raw reads need to be thoroughly filtered in order to produce clean reads. The steps for data preprocessing are as follows: (1) remove reads that are shorter than 55 base pairs (bp); (2) eliminate any adapter sequences present in the reads; (3) trim consecutive low-quality bases from both ends of the reads, specifically those with a quality value less than 19; (4) if the percentage of ambiguous bases (represented as 'N', which indicates indeterminate base information) in the single-ended sequencing reads exceeds 10%, it is necessary to discard that pair of reads to ensure accurate two-by-two matching and to output all matching results accordingly.

Analysis of reference sequence alignment and gene methylation results

Bismark was utilized to compare methylation data with the reference genome. It transformed the sequencing results and the reference genome by converting cytosine (C) to thymine (T) and guanine (G) to adenine (A), reflecting reverse complementarity. The transformed sequencing results were then compared to the genomic data in pairs, and the output was organized as follows: (1) the genome sequence and sequencing reads were converted from C to T and from G to A, respectively; (2) the converted reads were compared to the converted genomic DNA; (3) the best result from the four parallel comparisons was chosen as the final comparison outcome. To evaluate the degree of regional continuum methylation using paired-end reads (PE reads), the Methylation Haplotype Load (MHL) was calculated. This involved extracting C-site sequences from read pairs that covered the region of interest. Consecutive C strings were identified, and fully methylated strings were counted across i consecutive Cs. Each of these strings was assigned a weight, which was then summed to calculate a homogenization score by dividing the total methylated count by the summed weights [22]. The methylation detection values for each gene were obtained through the processing mentioned above for subsequent statistical analysis.

Statistical analysis

Pearson's chi-squared or Fisher's exact test was conducted to evaluate differences between categorical data groups. The diagnostic performance of each methylation marker was assessed using receiver operating characteristic (ROC) curves, focusing on metrics such as the area under the curve (AUC), sensitivity, specificity, and their respective 95% confidence intervals (CIs). Statistical analyses were performed using IBM SPSS Statistics 27 and GraphPad Prism 9, with significance defined as a *p*-value of less than 0.05.

Results

Characteristics of study participants

A total of 360 women were enrolled in this study and divided into five groups: control group (148 cases), CIN1 group (134 cases), CIN2 group (57 cases), CIN3 group (7 cases), and CC group (14 cases). The age range for the control group was 20 to 61 years, with a mean age of 34.03 ± 8.67 years. The CIN1 group had ages ranging from 19 to 68 years, with a mean age of 36.05 ± 9.76 years. The CIN2 group included individuals aged 22 to 59 years, with a mean age of 36.39 ± 10.04 years. The CIN3 group ranged in age from 29 to 51 years, with a mean age of 41.43 ± 9.45 years. Lastly, the CC group had ages between 29 and 63 years, with a mean age of 44.29 ± 10.77 years. Significant differences were observed in HPV16/18 positivity and cytology positivity rates among the groups (all $P < 0.001$, see Table 1).

Comparison of methylation levels and methylation positivity rates between different cervical lesion groups

Figure 3 shows the methylation positivity levels of various candidate genes in the control group, CIN1 group, CIN2 group, CIN3 group, and CC group. There were significant differences in methylation levels for all candidate genes between the control and CC groups (all $P < 0.0001$).

Table 1 Overview of general data characteristics ($n = 360$)

	Histological groups($n = 360$)					Total	χ^2	*P*-value
	Control group ($n = 148$)	CIN1 group ($n = 134$)	CIN2 group ($n = 57$)	CIN3 group ($n = 7$)	CC group ($n = 14$)			
Cytology results							98.32	< 0.001
NILM	53(35.8%)	49(36.6%)	26(45.6%)	1(14.3%)	0(0.0%)	129(35.8%)		
ASCUS	85(57.4%)	60(44.8%)	14(24.6%)	4(57.1%)	3(21.4%)	166(46.1%)		
LSIL	9(6.1%)	12(9.0%)	7(12.3%)	2(28.6%)	1(7.1%)	31(8.6%)		
HSIL/ASC-H	1(0.7%)	13(9.7%)	10(17.5%)	0(0.0%)	10(71.4%)	34(9.4%)		
hrHPV results							19.00	< 0.001
16 and/or 18 positive	78(52.7%)	88(65.7%)	45(78.9%)	5(71.4%)	13(92.9%)	229(63.6%)		
Other types of hrHPV-positive	70(47.3%)	46(34.3%)	12(21.1%)	2(28.6%)	1(7.1%)	131(36.4%)		

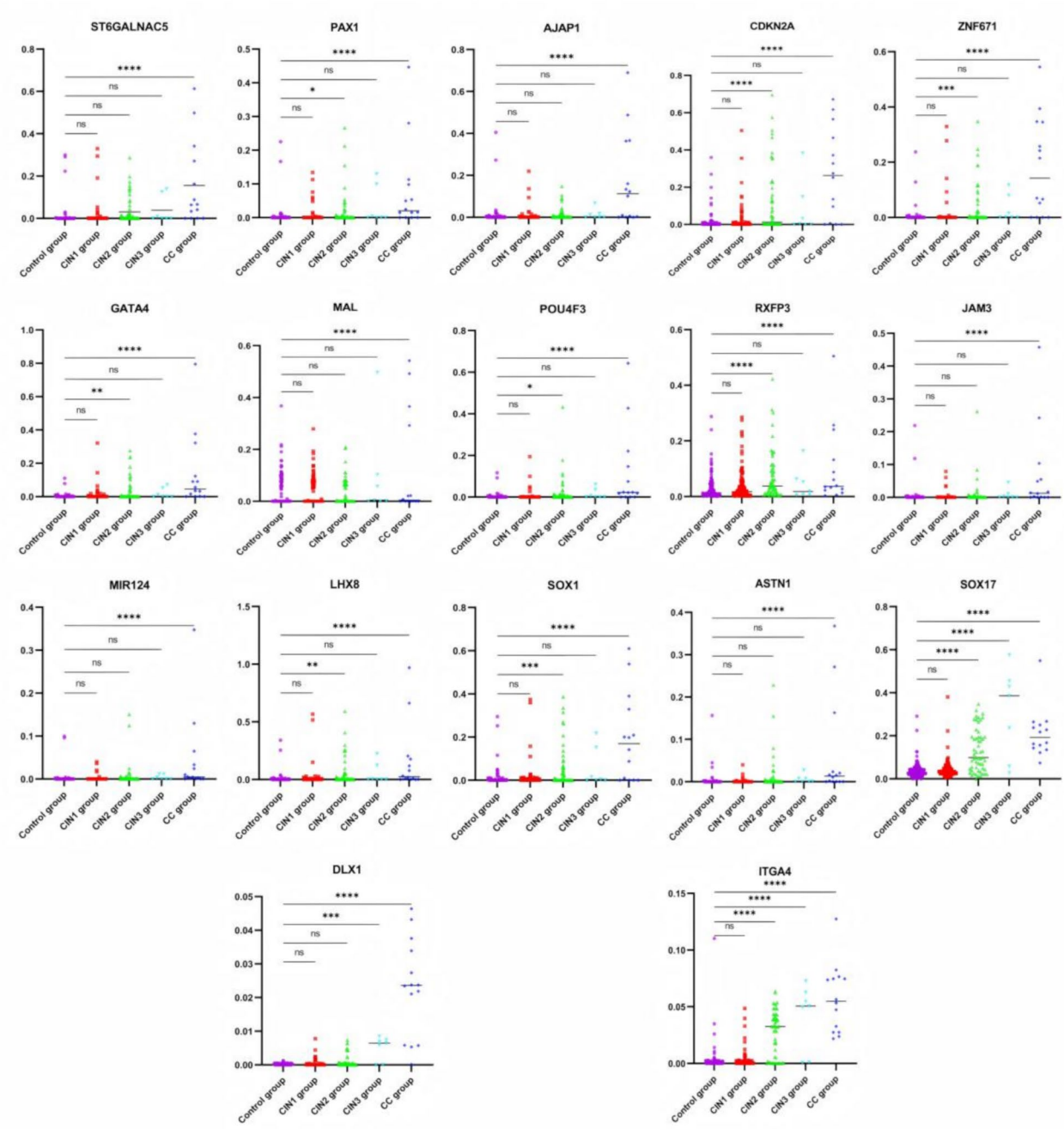

Fig. 3 Comparison of methylation levels between different groups of cervical lesions. ns: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$

The ITGA4 methylation assay showed that the methylation levels of the CIN2 group, CIN3 group, and CC group were significantly different from the control group (all $P < 0.0001$).

Table 2 shows the methylation positivity rates of various candidate genes in the control group, CIN1 group, CIN2 group, CIN3 group, and CC group. There were significant differences in methylation positivity rates for all candidate genes between groups (all $P < 0.001$).

Diagnostic performance of single and all candidate genes methylation detection for CIN2+

Figure 4 shows the ROC curve analysis of the diagnostic performance of each candidate gene methylation in CIN2 + detection, among which ITGA4 methylation testing shows an AUC of 0.866, indicating optimal performance in single gene methylation detection. In addition, the combined methylation test for all candidate genes shows an AUC of 0.915, which is better than any single

Table 2 Comparison of methylation rates between different groups of cervical lesions ($n=360$)

Methylation marker	The positive rate ($n=360$)					χ^2	*P*-value
	Control group ($n=148$)	CIN1 group ($n=134$)	CIN2 group ($n=57$)	CIN3 group ($n=7$)	CC group ($n=14$)		
ST6GALNAC5	4.1% (6)	6.0% (8)	36.8% (21)	42.9% (3)	71.4% (10)	90.75	< 0.001
PAX1	12.8% (19)	14.2% (19)	49.1% (28)	57.1% (4)	71.4% (10)	60.97	< 0.001
AJAP1	12.2% (18)	7.5% (10)	36.8% (21)	42.9% (3)	78.6% (11)	66.33	< 0.001
CDKN2A	6.1% (9)	11.2% (15)	45.6% (26)	42.9% (3)	71.4% (10)	79.58	< 0.001
ZNF671	7.4% (11)	7.5% (10)	40.4% (23)	57.1% (4)	71.4% (10)	80.86	< 0.001
GATA4	20.3% (30)	18.7% (25)	50.9% (29)	57.1% (4)	78.6% (11)	46.16	< 0.001
MAL	43.9% (65)	51.5% (69)	82.5% (47)	71.4% (5)	71.4% (10)	27.56	< 0.001
POU4F3	6.1% (9)	4.5% (6)	36.8% (21)	42.9% (3)	71.4% (10)	87.70	< 0.001
RXFP3	25.7% (38)	32.8% (44)	57.9% (33)	42.9% (3)	64.3% (9)	23.78	< 0.001
JAM3	19.6% (29)	16.4% (22)	45.6% (26)	57.1% (4)	71.4% (10)	40.13	< 0.001
MIR124	6.1% (9)	6.7% (9)	43.9% (25)	42.9% (3)	71.4% (10)	90.09	< 0.001
LHX8	13.5% (20)	17.2% (23)	47.4% (27)	42.9% (3)	71.4% (10)	49.22	< 0.001
SOX1	9.5% (14)	11.9% (16)	45.6% (26)	42.9% (3)	71.4% (10)	66.47	< 0.001
ASTN1	13.5% (20)	19.4% (26)	49.1% (28)	57.1% (4)	78.6% (11)	56.03	< 0.001
SOX17	7.4% (11)	9.7% (13)	66.7% (38)	71.4% (5)	100% (14)	155.45	< 0.001
DLX1	5.4% (8)	9.0% (12)	35.1% (20)	71.4% (5)	92.9% (13)	110.16	< 0.001
ITGA4	2.7% (4)	4.5% (6)	68.4% (39)	71.4% (5)	100.0% (14)	209.91	< 0.001

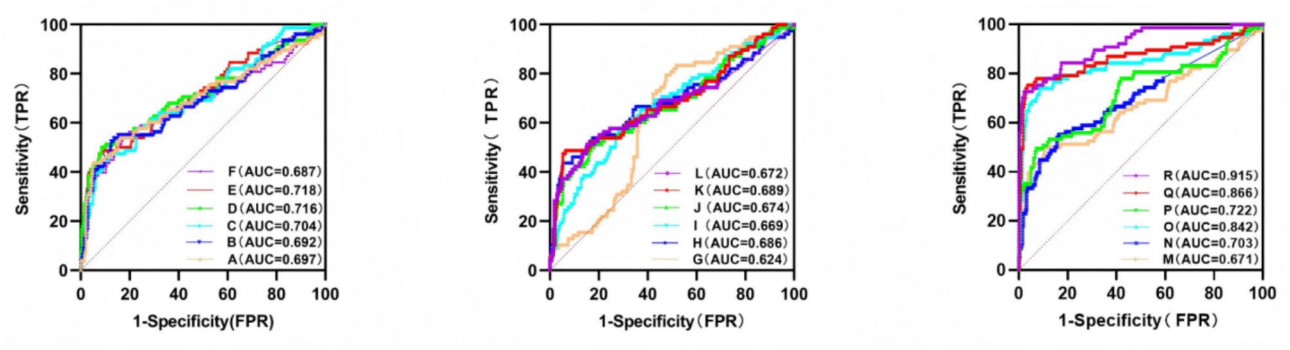

Fig. 4 ROC curve of the diagnostic efficacy of seventeen candidate gene methylations in CIN2 + detection. A: ST6GALNAC5; B: PAX1; C: AJAP1; D: CDKN2A; E: ZNF671; F: GATA4; G: MAL; H: POU4F3; I: RXFP3; J: JAM3; K: MIR124; L: LHX8; M: SOX1; N: ASTN1; O: SOX17; P: DLX1; Q: ITGA4; R: Combined detection of all candidate genes

gene methylation test. Table 3 indicates that all 17 genes demonstrated good specificity, with ITGA4 exhibiting the highest specificity at 96.45% (95% CI:0.936–0.981) and a sensitivity of 75.32% (95% CI:0.647–0.836). MAL exhibited the highest sensitivity at 79.49% (95% CI:0.693–0.870) but had a low specificity of 52.48% (95% CI:0.467–0.582). The combined methylation test for all candidate genes achieved a specificity of 97.87% (95% CI:0.954–0.990), surpassing that of any individual gene methylation test; however, its sensitivity was only 72.73% (95% CI: 0.619–0.814).

Comparison of HPV 16/18 testing, cytology, and gene methylation testing for the diagnosis of CIN2+

The sensitivity and specificity of the five detection methods were compared in Table 4. The diagnostic accuracy of HPV16/18 testing combined with all candidate genes methylation testing for the diagnosis of CIN2 + was significantly higher than that of HPV16/18 testing combined with cytology, with an AUC of 0.907 (95% CI:0.858–0.955), sensitivity of 72.73% (95% CI:0.619–0.814), and specificity of 98.58% (95% CI:0.964–0.995), respectively.

Discussion

Primary hrHPV-based screening has sufficient sensitivity for detecting high-grade CIN and cancer; however, its low specificity results in increased clinical workload and colposcopy referrals [23, 24]. Host-cell DNA methylation markers indicate the carcinogenic process and provide an opportunity to enhance cervical cancer screening and manage women with CIN, thereby preventing over-referral and over-treatment [25]. According to the latest U.S. guidelines released in 2020, all cases of CIN2 and higher should be treated unless the risk of complications during

Table 3 Comparison of the diagnostic performance of eighteen detection methods for CIN2+

Detection Method	Cut-off value	AUC	Sensitivity	Specificity	Youden index	95% CI
ST6GALNAC5	0.00413	0.697	43.59%	95.04%	0.379	0.621–0.773
PAX1	0.00060	0.692	53.85%	86.52%	0.398	0.617–0.766
AJAP1	0.00320	0.704	44.87%	90.07%	0.355	0.634–0.774
CDKN2A	0.02831	0.716	50.00%	91.49%	0.408	0.642–0.790
ZNF671	0.00107	0.718	47.44%	92.55%	0.393	0.648–0.788
GATA4	0.00154	0.687	56.41%	80.50%	0.363	0.610–0.764
MAL	0.00011	0.624	79.49%	52.48%	0.330	0.560–0.689
POU4F3	0.00283	0.686	43.59%	94.68%	0.375	0.608–0.764
RXFP3	0.03170	0.669	57.69%	70.92%	0.281	0.599–0.739
JAM3	0.00076	0.674	51.28%	81.91%	0.326	0.600-0.748
MIR124	0.00020	0.689	48.72%	93.62%	0.417	0.615–0.764
LHX8	0.00296	0.672	51.28%	84.75%	0.354	0.593–0.751
SOX1	0.00697	0.671	50.39%	89.36%	0.387	0.592–0.749
ASTN1	0.00018	0.703	55.13%	83.69%	0.382	0.630–0.777
SOX17	0.06760	0.842	74.03%	91.49%	0.655	0.779–0.905
DLX1	0.00048	0.722	49.35%	92.91%	0.423	0.647–0.796
ITGA4	0.01130	0.866	75.32%	96.45%	0.718	0.806–0.925
Combined detection of all candidate genes	0.48360	0.915	72.73%	97.87%	0.706	0.877–0.954

Table 4 Comparison of the diagnostic performance of five detection methods for CIN2+

Detection Method	AUC	Sensitivity	Specificity	Youden index	95% CI
HPV16/18 testing	0.610	80.77%	41.13%	0.217	0.543–0.677
Cytology	0.679	38.46%	87.59%	0.265	0.607–0.750
Combined detection of HPV16/18 and cytology	0.709	37.18%	92.55%	0.302	0.641–0.777
Combined detection of HPV16/18 and ITGA4	0.880	77.92%	94.33%	0.722	0.826–0.934
Combined detection of HPV16/18 and all candidate genes	0.907	72.73%	98.58%	0.713	0.858–0.955

future pregnancies outweighs the risk of developing cancer [6]. As a result, our study analyzed 360 hrHPV-positive cases and provided comprehensive histopathological data to evaluate the triage accuracy of 17 candidate methylation genes, either alone or in combination, for detecting CIN2 + in relation to hrHPV triage. We found that the positivity rate of methylated genes was significantly higher in CIN2 + lesions, indicating a strong triage value.

A 2021 study [26] evaluated the performance of a panel of six methylation markers (ASTN1, DLX1, ITGA4, RXFP3, SOX17, and ZNF671), showing a sensitivity of 45.5% (95% CI: 0.27–0.65) and a specificity of 78.3% (95% CI: 0.64–0.88) for identifying CIN2+. A recent study conducted in 2023 examined the effectiveness of detecting PAX1 and SEPT9 methylation for diagnosing cervical pre-cancer and cervical cancer. The AUC for PAX1 and SEPT9 were found to be 0.77 (95% CI: 0.71–0.83) and 0.86 (95% CI: 0.81–0.90), respectively [7]. A 2024 study [27] found that detecting PAX1/SOX1 gene methylation demonstrates strong diagnostic efficacy for cervical precancer and holds significant value in triage diagnosis. Based on the above, DNA methylation testing is expected to be a biomarker for triaging cervical lesions. However, the clinical manifestations of DNA methylation vary in different population studies, and different study groups in different populations with different analytical methods have conducted most of the studies based on DNA methylation biomarkers. We investigated the methylation patterns of 17 candidate genes (ST6GALNAC5, PAX1, AJAP1, CDKN2A, ZNF671, GATA4, MAL, POU4F3, RXFP3, JAM3, MIR124, LHX8, SOX1, ASTN1, SOX17, DLX1, and ITGA4) in the same population and found that each candidate gene exhibited high diagnostic performance for detecting CIN2+. Among these genes, ITGA4 methylation testing showed the highest diagnostic efficacy for CIN2+, with an AUC of 0.866 (95% CI: 0.806–0.925). This test had a sensitivity of 75.32% (95% CI:0.647–0.836) and a specificity of 96.45% (95% CI: 0.936–0.981). We were pleasantly surprised that ITGA4 gene methylation testing may be the most effective gene methylation detection for triaging CIN2 + in hrHPV-positive patients among these 17 candidate genes. Our findings differ somewhat from those of the studies mentioned earlier, which may be attributed to differences in the study populations and the methods used for gene methylation analysis. Furthermore, the combined methylation test for all candidate genes demonstrated a specificity of 97.87% (95% CI:0.954–0.990), surpassing any individual gene methylation test. However, this combined

test achieved a lower sensitivity of 72.73% (95% CI: 0.619–0.814).

In addition, our study analyzed HPV16/18 testing combined with all candidate genes methylation testing for triaging CIN2 + in hrHPV-positive patients. We found the diagnostic efficiency was significantly higher than that of HPV16/18 testing combined with cytology, with an AUC of 0.907 (95% CI:0.858–0.955), sensitivity of 72.73% (95% CI:0.619–0.814), and specificity of 98.58% (95% CI:0.964–0.995), respectively.

Our study has several limitations that should be acknowledged. Selection bias may have occurred because all patients were from the same hospital, and the sample size for CIN2 + cases was relatively small. Furthermore, because cervical histopathologic findings are regarded as the gold standard for diagnosing cervical lesions, our study only included women who underwent cervical biopsy. This inclusion criterion may have resulted in a higher proportion of cervical lesions among the control group and an increased rate of CIN2 + in the NILM cases. While multigene methylation testing offers more thorough insights than single-gene testing, its greater cost and complexity should be considered for practical application. Although multigene methylation testing provides better diagnostic accuracy than hrHPV combined with cytology, its significantly higher cost restricts its practical use. Additionally, our study focused solely on the methylation status of 17 candidate genes without exploring their underlying mechanisms, and we did not have follow-up data.

Future research should aim to increase the sample size, include hrHPV patients without cervical lesions observed during colposcopy, conduct prospective observational studies, and assess both the economic and clinical diagnostic utility of methylation testing.

Conclusion

Multigene methylation testing is an efficient triage test for CIN2 + in hrHPV-positive patients and has potential value in clinical practice. Combined HPV16/18 and multigene methylation testing for the triage of CIN2 + is significantly better than combined HPV16/18 and cytology testing.

Abbreviations

DNA	Deoxyribonucleic acid
ST6GALNAC5	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
PAX1	Paired box 1
AJAP1	Adherens junctions associated protein 1
CDKN2A	Cyclin-dependent kinase inhibitor 2 A
ZNF671	Zinc finger protein 671
GATA4	GATA binding protein 4
MAL	T-lymphocyte maturation-associated protein
POU4F3	POU class 4 homeobox 3
RXFP3	Relaxin family peptide receptor 3
JAM3	Junctional Adhesion Molecule 3
MIR124	MicroRNA 124
LHX8	LIM homeobox 8
SOX1	SRY-box transcription factor 1
ASTN1	Astrotactin 1
SOX17	SRY-box transcription factor 17
DLX1	Distal-less homeobox 1
ITGA4	Integrin subunit alpha 4
ZNF582	Zinc finger protein 582
FAM19A4	Family with sequence similarity 19 member A4
CADM1	Cell adhesion molecule 1
SEPT9	Septin 9
ASCL1	Achaete-scute complex homolog 1

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Author contributions

CY. L. contributed to study design, data collection, data analysis, original manuscript drafting, and funding acquisition. CY. Z. was responsible for patient recruitment and specimen collection. MH. X. contributed to the study design and drafting of the manuscript. H. Y. contributed to study design, data curation, manuscript revision, and funding acquisition. All authors reviewed the manuscript.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics statement

This study received approval from the Medical Ethics Committee of Hainan Women and Children's Medical Center (Ethics Number: HNWCMC MEC No. 095 of 2022), China, and it was conducted in adherence to the principles of the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare no conflict of interest.

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