BMC Cancer

RESEARCH

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Comparing the performance of DeoxyriboNucleic Acid methylation analysis and cytology for detecting cervical (pre)cancer in women with high-risk human papillomavirus-positive status in a gynecologic outpatient population

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Abstract

Background Primary screening for high-risk human papillomavirus (hrHPV) with cytological triage for women with non-16/18 hrHPV-positive status has become popular in China. However, cytology relies on the subjective judgment of pathologists, leading to inconsistent clinical performance.

Methods A total of 657 hrHPV-positive women aged 25–64 years were enrolled in this cross-sectional study. All participants underwent colposcopic biopsy after cytology triage, with cytology residual specimens undergoing DNA methylation testing. CIN2+ and CIN3+ sensitivity and specificity were compared between the different triage strategies (n=487): *PAX1* methylation ($PAX1^m$) , Glycophorin C methylation ($GYPC^m$), cytology, and combinations between them or with HPV16/18.

Results The area under the receiver operating characteristic curves (AUCs) for $PAX1^m$ and $GYPC^m$ in detecting CIN2 or worse (CIN2+) were 0.867 (95% confidence interval [CI]: 0.796–0.937) and 0.873 (95% CI: 0.808–0.938), respectively. The sensitivities of $PAX1^m$ and $GYPC^m$ were consistent with those of cytology for both CIN2+ and CIN3+ detection. The relative specificities of $PAX1^m$ and $GYPC^m$ for CIN2+ detection compared to cytology were 2.83 (95% CI: 2.33–2.45) and 3.09 (95% CI: 2.40–3.98), respectively. The relative specificities of combining HPV 16/18 with $PAX1^m$ and $GYPC^m$ for CIN2+ detection compared to cytology were 3.38 (95% CI: 2.96–3.86) and 3.67 (95% CI: 3.15–4.27), respectively. Compared to low levels of DNA methylation, high levels of $PAX1^m$ and $GYPC^m$ resulted in odd ratios (ORs) of 57.66 (95% CI: 13.57–409.12, $p < 0.001$) and 23.87 (95% CI: 6.49–115.42, $p < 0.001$) for CIN3+, adjusted for HPV 16/18 and cytology results.

Conclusions $PAX1^m$ and $GYPC^m$ demonstrated superior ability to identify cervical precancerous lesions and cervical cancer, with AUC values exceeding 0.85. For detecting CIN2+/CIN3+ in women with hrHPV-positive status, DNA

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methylation (combined with HPV 16/18) showed higher specificity than cytology (combined with HPV 16/18) and is a potential molecular biomarker for detecting cervical (pre)cancer.

Keywords *PAX1* methylation, *GYPC* methylation, DNA methylation, Cervical cancer, Cervical intraepithelial neoplasia, High-risk human papillomavirus, Relative sensitivity, Relative specificity

Background

Generally, hrHPV-DNA testing is widely recognized as the primary method for screening cervical (pre)cancer, having replaced cytological examination as the primary screening approach [1]. Persistent hrHPV infection is the primary cause of cervical cancer (CC) [2], with hrHPV testing exhibiting high sensitivity and a negative predictive value for high-grade cervical lesions and CC [3, 4]. Thus, multiple national and regional guidelines recommend hrHPV as the primary screening test for CC [5].

Most hrHPV-positive cases represent transient HPV infections and do not lead to related diseases. Without an appropriate triage strategy, direct referral of all hrHPV cases can result in an unacceptably high rate of vaginal colposcopy, causing anxiety and unnecessary treatments [6]. Triage tests for patients with hrHPV-positive status include HPV 16/18 genotyping, cytology, and p16/Ki-67 cytoimmunochemistry [7]. Among these, the combination of HPV 16/18 and cytology is essential, as it helps reduce the colposcopy referral rate [8]. However, the subjective nature of cytology and the low threshold for atypical squamous cells of undetermined significance (ASC-US) referrals result in many patients undergoing unnecessary colposcopy examinations. The limitation of HPV 16/18 genotyping is that other hrHPV types can also cause serious related diseases. Additionally, with the increasing number of individuals vaccinated against HPV 16/18, the incidence of cervical lesions associated with HPV 16/18 has declined, potentially reducing the effectiveness of this genotyping method [9].

The methylation of host DNA has been shown to have high sensitivity and specificity for cervical intraepithelial neoplasia or worse (CIN2+), especially for invasive cancers [10–14]. The *PAX1* gene is a tumor suppressor gene that inhibits the malignant phenotype of cells under the carcinogenic pressure of hrHPV. It activates dual specificity phosphatases 1, 5, and 6, inhibiting the EGF/MAPK signaling pathway to suppress cancer [15]. However, the *PAX1* gene often becomes abnormally hypermethylated, silenced, and inactivated, losing its tumor-suppressive function [16, 17]. The application of $PAX1^m$ in CC screening and prevention is as follows: (1) triaging women with non-16/18 hrHPV-positive status [18]; (2) predicting the progression of cervical lesions after CIN2/CIN3 conization [19]; (3) assessing its relationship with hrHPV load and p16/Ki67 immunohistochemical staining [19, 20]; (4) assessing the necessity of cervical canal curettage (ECC) [21]; (5) monitoring CC treatment [22]; and (6) prognostic evaluation of CC [23]. The *GYPC* gene for the human erythrocyte membrane glycoprotein C, also known as glycophorin C, is located on human chromosome 2 and contains multiple exons and introns that encode a protein containing 128 amino acid residues. Significant methylation folding changes in *GYPC* have been observed in the plasma of patients with ovarian cancer compared to that in the plasma patients without ovarian cancer [24]. To our knowledge, limited research exists on the correlation between Glycophorin C methylation ($GYPC^m$) and CC. $GYPC^m$ combined $ZSCAN12^m$ can be used for diagnosing uterine cancers with a sensitivity of 90.9% [25].

In this study, we explored the clinical performance of DNA methylation and compared triage strategies for detecting cervical (pre)cancer in women with hrHPV-positive status undergoing outpatient opportunistic cervical screening. We assessed the performance of DNA methylation markers, $PAX1^m$ and $GYPC^m$, both alone and in combination with cytology and HPV 16/18 testing.

Methods

Study population and sample collection

This cross-sectional study included women who tested positive for hrHPV during CC screening at hospital outpatient clinics from June to December 2023. The inclusion criteria were as follows: (1) age 25–64 years, (2) not pregnant or lactating, (3) no history of surgical resection for cervical lesions or CC, (4) no history of other cancers, (5) HIV negative, and (6) normal immune function. The exclusion criteria were as follows: (1) samples with insufficient liquid-based cells or ineffective DNA methylation detection, and (2) those who had not completed the pathological diagnosis of colposcopy-directed biopsy.

The specimens used in this study were cervical exfoliated cells collected by a colposcopy physician. Liquid-based cytology (LBC) was conducted first, followed by DNA extraction from the remaining specimens. These specimens were stored at −20℃ and subjected to DNA methylation testing after obtaining the pathological results. The study flow chart is shown in Fig. 1. This study was approved by the Research and Clinical Trial Ethics Committee of Changsha Hospital for Maternal & Child Health Care (No. EC-20230726-02), and all participants

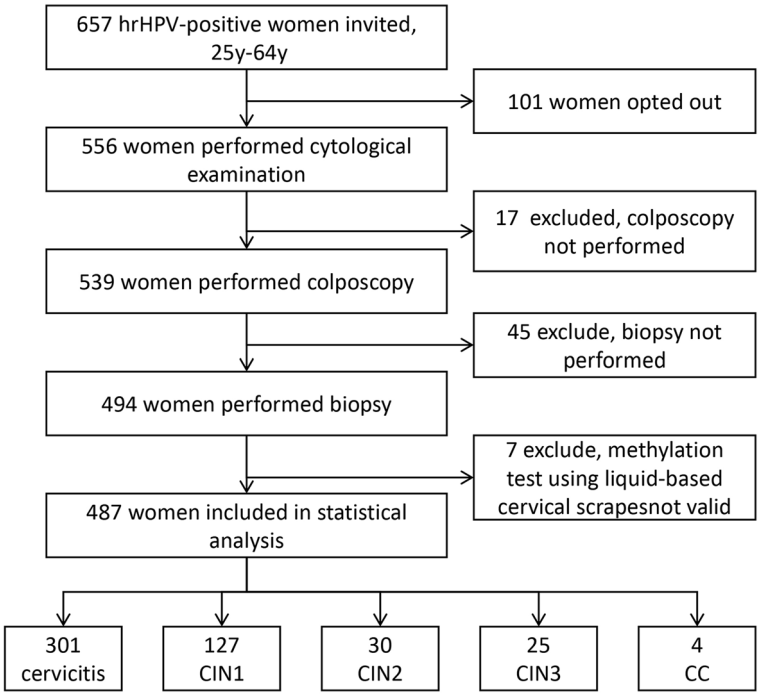

Fig. 1 Study flow chart. hrHPV, high-risk Human papillomavirus; CIN1, Cervical intraepithelial neoplasia grade 1; CIN2, Cervical intraepithelial neoplasia grade 2; CIN3, Cervical intraepithelial neoplasia grade 3; CC, Cervical cancer

provided written informed consent before specimens collection.

HrHPV testing

HPV testing was conducted using 21 subtypes (HybriBio Ltd., Guangzhou, China) following the manufacturer's instructions. The simple procedure involves: (1) amplification of HPV DNA by PCR, (2) hybridization of the DNA amplicon with fixed specificity using HybriBio's proprietary flow hybridization technology, and (3) identification of 21 HPV genotypes by enzyme immunoassay. This study included women who tested positive for 14 hrHPV types and one potentially high-risk type (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68).

Cytology

Those with hrHPV-positive status were advised to undergo cytological examination for triage. Participants were instructed to avoid sexual intercourse for 1 d before sampling and to refrain from vaginal flushing or medication for 3 d before sampling. Cytological sampling was performed at least 5 d after menstruation. Cervical exfoliated cells were preserved in a liquid cell preservation solution (Hologic, MA, USA). Subsequently, the collected cells were processed into thin smears using a ThinPrep cytology analyzer, examined under a microscope, and interpreted according to the 2014 Bethesda system guidelines.

Colposcopy-directed biopsy

For women referred for colposcopy, 1–3 samples of living tissue were taken from the site of the most severe cervical lesion. Four specimens were randomly selected in a counterclockwise direction in cases with a normal colposcopic impression. Biopsy specimens were stained with hematoxylin and eosin and sectioned for initial examination by a pathologist, followed by an independent secondary examination by another pathologist. In cases where the first two assessments were inconsistent, a third pathologist was consulted to make the final determination.

PAX1 and GYPC methylation analysis

The remaining DNA from cervical exfoliated cells were stored at −20℃ and subjected to DNA methylation testing within 3 months. This testing was conducted at a certified laboratory in China (Hoomya Medical Laboratory, Changsha). $PAX1^m$ and $GYPC^m$ analysis were performed using the cervical cancer gene methylation test kit (Hoomya) according to the manufacturer's instructions. The COL2A1 gene served as an internal reference, with methylation results calculated as $\Delta Cp_{gene} = Cp_{gene} - Cp_{COL2A1}$. The DNA methylation analysis involved the following steps: (1) bisulfite conversion, which transforms

unmethylated cytosine (C) into uracil (U) while leaving methylated cytosine unchanged; (2) amplification of 50 cycles using a fluorescence quantitative polymerase chain reaction instrument (LC480; Roche Applied Science, CA, USA); and (3) calculation of ΔCp of the target gene. When the quality control was met, but the target gene was not amplified, the Cp of the target gene was set to 50, and ΔCp calculation was performed.

Statistical analysis

All data were analyzed using R Version 4.3.2. Two-sided *p*-values < 0.05 were considered statistically significant. Continuous variables, such as age, $PAX1^m$, and $GYPC^m$, were nonnormally distributed and are presented as median (interquartile range, IQR). Categorical variables were recorded as frequencies and percentages. The distribution plots of $PAX1^m$ and $GYPC^m$ in cervical lesions were generated using the "ggplot2" package, while receiver operating characteristic (ROC) curves were constructed using the "pROC" package. To maximize the Youden index for diagnosing CIN2+, the criteria for determining positive results for $PAX1^m$ and $GYPC^m$ were set at ΔCp ≤ 10.86 and ΔCp ≤ 5.97, respectively. Conversely, negative results were ΔCp > 10.86 for $PAX1^m$ and ΔCp > 5.97 for $GYPC^m$. The sensitivity and specificity of each ΔCp value of $PAX1^m$ and $GYPC^m$ were obtained from ROC analysis (Supplementary Tables 1and 2). Using the same principle for detecting CIN2+ and CIN3+, $PAX1^m$ was classified into three levels using two cut-off values as follows: low (ΔCp > 11), moderate (9 < ΔCp ≤ 11), and high (ΔCp ≤ 9). $GYPC^m$ was similarly categorized into low (ΔCp > 6), moderate (4 < ΔCp ≤ 6), and high (ΔCp ≤ 4). The calculation of sensitivity and specificity was performed using the "gmodels" and "DescTools" packages, with the Wilson score method used to estimate 95% confidence intervals (CIs). The 95% CIs for relative sensitivity and specificity were calculated using the formula proposed by Hayen et al. [26]. Odds ratios (ORs) and adjusted ORs for $PAX1^m$ and $GYPC^m$ levels were estimated using logistic regression.

Results

Patients and histological outcomes

This study included 487 women with hrHPV-positive status, with a median age of 42 years (IQR: 34.5–51.0). Of these, 381 women (78.2%) were infected with one hrHPV subtype, while 7 women (1.4%) were infected with four or more hrHPV subtypes. The positivity rate for HPV 16/18 was 17.9% (87 cases). The rate of cytological abnormalities (≥ ASC-US) was 73.3%. The median ΔCp value for $PAX1^m$ was 13.9 (10.4–20.4), and for $GYPC^m$ was 14.0 (6.8–20.3). Histological outcomes included 301 cases of cervicitis (61.8%), 127 cases of CIN1 (26.1%), 30 cases of CIN2 (6.2%), 25 cases of CIN3 (5.1%), and 4 cases of CC (0.8%) (Table 1).

Distribution and AUCs of $PAX1^m$ and $GYPC^m$

The distribution of ΔCp values for $PAX1^m$ and $GYPC^m$ in histopathological and cytological results is shown in Fig. 2A–D. Statistically significant differences were observed in the ΔCp values of $PAX1^m$ and $GYPC^m$ between the LSIL and ASC-H/AGC groups (all $p < 0.01$). The differences in $PAX1^m$ ΔCp values between CIN1 and CIN2, as well as between CIN2 and CIN3, were statistically significant (all $p < 0.01$). A statistically significant difference in $GYPC^m$ ΔCp values was also observed between CIN1 and CIN2 ($p < 0.001$). The area under the ROC curve (AUC) values for detecting CIN2+ with $PAX1^m$ and $GYPC^m$ were 0.867 (95% CI: 0.796–0.937) and 0.873 (95% CI: 0.808–0.973), respectively (Fig. 2E). The AUCs of triage strategies for detecting CIN2+ were

Table 1 Basic characteristic of participants

	level	Percentage
n		487
Age (median [IQR])		42.0 [34.5, 51.0]
HPV infection (%)	Single	381 (78.2)
	Double	79 (16.2)
	Triple	20 (4.1)
	Quadruple	4 (0.8)
	Quintuple	3 (0.6)
HPV16/18 (%)	Negative	400 (82.1)
	Positive	87 (17.9)
Cytology (%)	NILM	130 (26.7)
	ASC-US	218 (44.8)
	LSIL	90 (18.5)
	ASC-H/AGC	36 (7.4)
	HSIL	13 (2.7)
***PAX1* methylation (median [IQR])**		13.9 [10.4, 20.4]
GYPC methylation (median [IQR])		14.0 [6.8, 20.3]
Colposcopy (%)	Normal	6 (1.2)
	LSIL	424 (87.1)
	HSIL	56 (11.5)
	CC	1 (0.2)
Pathology (%)	Cervicitis	301 (61.8)
	CIN1	127 (26.1)
	CIN2	30 (6.2)
	CIN3	25 (5.1)
	CC	4 (0.8)

Abbreviation: IQR Interquartile range, *HPV* Human papillomavirus, *NILM* No intraepithelial lesions or malignancy, *ASC-US* Atypical squamous cells of undetermined significance, *LSIL* Low-grade squamous intraepithelial lesion, *ASC-H* Atypical squamous cells cannot exclude HSIL, *AGC* Atypical glandular cells, *HSIL* High-grade squamous intraepithelial lesion, *CC* Cervical cancer, CIN1 Cervical intraepithelial neoplasia grade 1, *CIN2* Cervical intraepithelial neoplasia grade 2, *CIN3* Cervical intraepithelial neoplasia grade 3

detailed in Supplementary Table 3. The AUC values for detecting CIN3+ with $PAX1^m$ and $GYPC^m$ were 0.910 (95% CI: 0.830–0.990) and 0.896 (95% CI: 0.818–0.974), respectively (Fig. 2F), showing good discriminative ability.

Performance of triage markers for CIN2+ and CIN3+ detection

The sensitivity for detecting CIN2+ and CIN3+ in women with hrHPV-positive status using cytological triage was 84.7% (95% CI: 73.5%–91.8%) and 89.7% (95% CI: 73.6%–96.4%), respectively. The sensitivities of $PAX1^m$ and $GYPC^m$ were consistent with those of cytology, with relative sensitivities of 1.04 (95% CI: 0.52–2.09) and 1.02 (95% CI: 0.55–1.88) for detecting CIN2+, and 1.04 (95% CI: 0.26–4.15) and 1.00 (95% CI: 0.40–2.52) for detecting CIN3+, respectively. The relative cytological specificities for $PAX1^m$ and $GYPC^m$ in detecting CIN2+ were 2.83 (95% CI: 2.33–2.45) and 3.09 (95% CI: 2.40–3.98), respectively, while for detecting CIN3+, the relative specificities were 2.74 (95% CI: 2.32–3.24) and 2.98 (95% CI: 2.43–3.66), respectively.

The sensitivity for detecting CIN2+ and CIN3+ in women with hrHPV-positive status using HPV 16/18 combined with cytology triage was 91.5% (95% CI: 81.6%–96.3%) and 96.6% (95% CI: 82.8–99.4), respectively. The sensitivities of $PAX1^m$ and $GYPC^m$ combined with cytology were consistent with those of HPV 16/18 combined with cytology, with relative sensitivities of 1.01 (95% CI: 0.38–2.71) and 1.00 (95% CI: 0.38–2.61) for detecting CIN2+, and 1.00 (95% CI: 0.37–2.67) and 0.96 (95% CI: 0.24–3.86) for detecting CIN3+, respectively. The relative specificities of $PAX1^m$ and $GYPC^m$ combined with cytology for detecting CIN2+ were 1.21 (95% CI: 1.17–1.26) and 1.28 (95% CI: 1.24–1.33), respectively. The relative specificities for detecting CIN3+ was 1.19 (95% CI: 1.14–1.23) and 1.26 (95% CI: 1.21–1.30), respectively.

The sensitivity of combining HPV 16/18 with $PAX1^m$ and $GYPC^m$ for detecting CIN2+ and CIN3+ was consistent, at 93.2% (95% CI: 83.8%–97.3%) and 96.6% (95% CI: 82.8%–99.4%), respectively. The relative specificities of HPV 16/18 combined with $PAX1^m$ and $GYPC^m$ for detecting CIN2+ were 3.38 (95% CI: 2.96–3.86) and 3.67 (95% CI: 3.15–4.27), respectively. For detecting CIN3+, the relative specificities were 3.26 (95% CI: 2.89–3.67) and 3.54 (95% CI: 3.10–4.05), respectively.

Whether alone or in combination, the ability of DNA methylation to detect CIN2+ and CIN3+ due to cytology is mainly manifested in higher specificity.

ORs of PAX1 and GYPC methylation levels for CIN2+ and CIN3+

The ORs for moderate and high levels of $PAX1^m$ compared to low levels for CIN2+ were 7.25 (95% CI: 2.69–20.61) and 97.14 (95% CI: 41.24–261.33), respectively. For CIN3+, the ORs were 4.60 (95% CI: 0.54–38.83) and 113.49 (95% CI: 32.14–722.80), respectively. The ORs for moderate and high levels of $GYPC^m$ compared to low levels for CIN2+ were 21.31 (95% CI: 8.75–55.94) and 81.59 (95% CI: 35.11–211.49), respectively. For CIN3+, the ORs were 23.44 (95% CI: 6.51–110.10) and 61.30 (95% CI: 19.64–270.53), respectively.

After adjusting for HPV 16/18 and cytological results, the ORs for high levels of $PAX1^m$ compared to low levels for CIN2+ and CIN3+ were 97.14 (95% CI: 41.24–261.33) and 113.49 (95% CI: 32.14–722.80), respectively. The ORs high levels of $GYPC^m$ compared to low levels for CIN2+ and CIN3+ were 45.95 (95% CI: 18.52–125.05) and 23.87 (95% CI: 6.49–115.42), respectively.

$GYPC^m$ is associated with a higher risk of CIN2+ and CIN3+ at moderate levels, while $PAX1^m$ is associated with a higher risk at high levels.

Discussion

This cross-sectional study indicates that molecular DNA methylation analysis is comparable to cytological examination regarding sensitivity for detecting CI2+ and CIN3+ in outpatient women with hrHPV-positive status aged 25–64 years. Specifically, the sensitivity for CIN2+ was approximately 85%for $PAX1^m$, $GYPC^m$ and cytology. For CIN3+, the sensitivities were approximately 90%. Notably, the specificity of DNA methylation (> 80%) for CIN2+ was significantly higher than that of cytology (approximately 30%). For CIN3+, the specificity of DNA

(See figure on next page.)

Fig. 2 Violin plots of *PAX1* and *GYPC* methylation distribution in lesions and ROCs plot for detection of CIN2+ and CIN3+. **A**. Distribution of *PAX1* methylation in histopathologic lesions. **B**. Distribution of *GYPC* methylation in cytopathologic lesions. **C**. Distribution of *PAX1* methylation in cytological lesions. **D**. Distribution of *GYPC* methylation in cytological lesions. **E**. ROC of GYPC methylation for detecting CIN2+. E. ROCs of *PAX1* and *GYPC* methylation for detecting CIN2+. **F**. ROCs of *PAX1* and *GYPC* methylation for detecting CIN3+. CIN1, Cervical intraepithelial neoplasia grade 1; CIN2, Cervical intraepithelial neoplasia grade 2; CIN3, Cervical intraepithelial neoplasia grade 3; CC, Cervical cancer; NILM, No intraepithelial lesions or malignancy; ASC-US, Atypical squamous cells of undetermined significance; LSIL, Low-grade squamous intraepithelial lesion; ASC-H, Atypical squamous cells cannot exclude HSIL; AGC, Atypical glandular cells; HSIL, High-grade squamous intraepithelial lesion; ROC, Receiver operating characteristic curve; AUC, Area under the curve; CIN2+, Cervical intraepithelial neoplasia grade 2 or worse; CIN3+, Cervical intraepithelial neoplasia grade 3 or worse. NS., no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

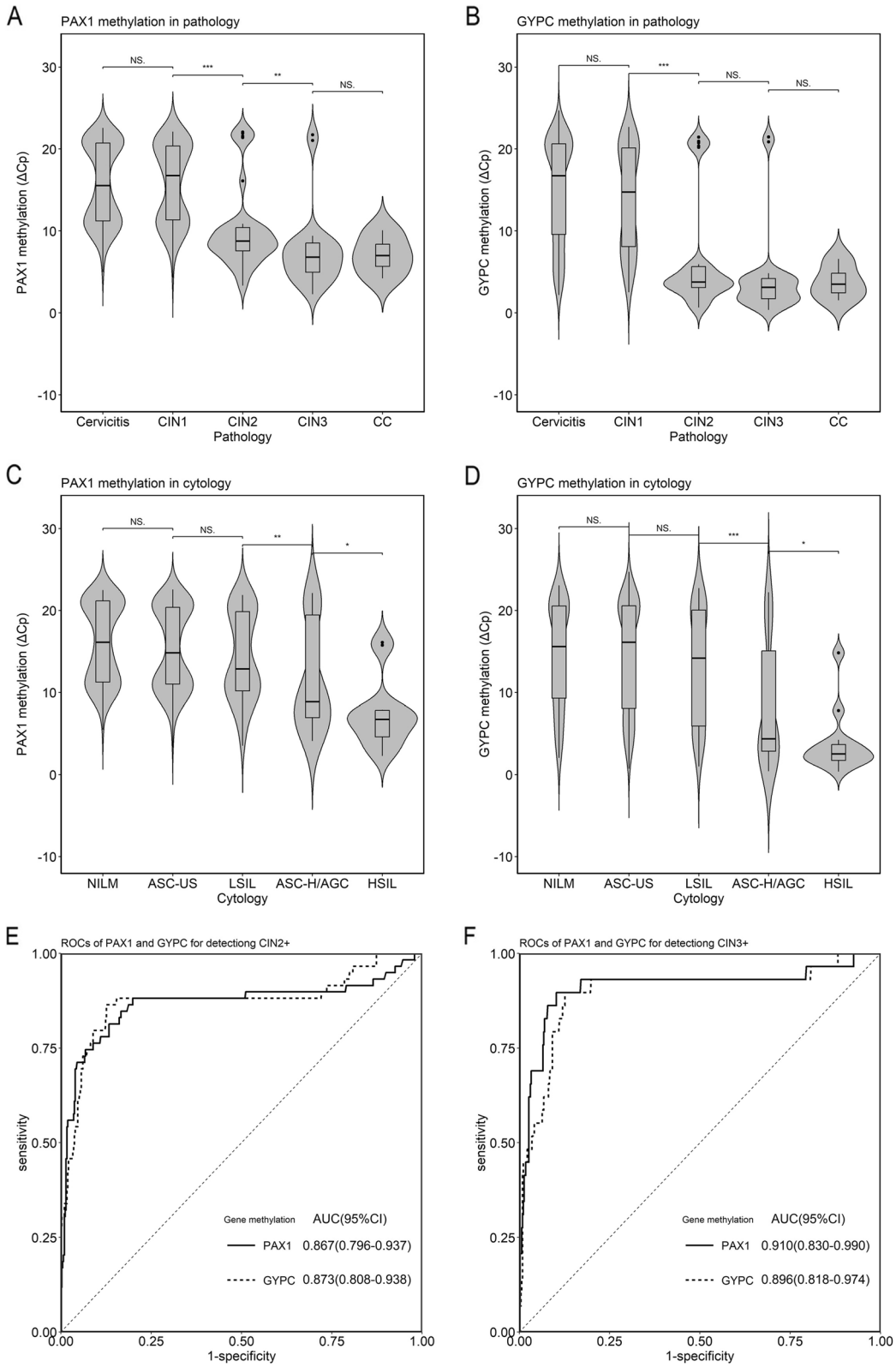

Fig. 2 (See legend on previous page.)

methylation (> 75%) was also higher than that of cytology (Table 2). Previous studies have shown that with an ASC-US threshold for cytology, the sensitivity is higher and does not significantly differ from that of DNA methylation. However, the specificity of DNA methylation was much higher than that of cytology [13, 27–29]. Among the abnormal cytological results, ASC-US accounted for 44.8%, and 206 cases (94.5%) of 218 ASC-US cases were NILM and CIN1, which may be the reason for the high cytological sensitivity and low specificity (Supplementary

Table 2 Sensitivities and specificities of different markers for CIN2+ and CIN3+ detection in hrHPV-positive women.

Triage marker	Sensitivity (%) (n/N) 95%CI	Specificity (%) (n/N) 95%CI	PPV (%) (n/N) 95%CI	NPV (%) (n/N) 95%CI	Compared with LBC ASC-US		Compared with HPV 16/18 or LBC ASC-US	
					Relative sensitivity (95%CI)	Relative specificity (95%CI)	Relative sensitivity (95%CI)	Relative specificity (95%CI)
For detecting CIN2+								
LBC ASC-US	84.7 (50/59) 73.5-91.8	28.3 (121/428) 24.2-32.7	14.0 (50/357) 10.8-18.0	93.1 (121/130) 87.4-96.3	1	1	—	—
HPV16/18	27.1 (16/59) 17.4-38.6	83.4 (357/428) 79.6-86.6	18.4 (16/87) 11.6-27.8	89.2 (357/400) 85.8-91.9	0.32 (0.17-0.61)	2.95 (2.34-3.73)	—	—
PAX1^m	88.1 (52/59) 77.5-94.1	80.1 (343/428) 76.1-83.7	38.0 (52/137) 30.3-46.3	98.0 (343/350) 95.9-99.0	1.04 (0.52-2.09)	2.83 (2.33-2.45)	—	—
GYPC^m	86.4 (51/59) 75.5-93.0	87.4 (374/428) 83.9-90.2	48.6 (51/105) 39.2-58.0	97.9 (374/382) 95.9-98.9	1.02 (0.55-1.88)	3.09 (2.40-3.98)	—	—
HPV 16/18 or LBC ASC-US	91.5 (54/59) 81.6-96.3	19.9 (85/428) 16.3-23.9	13.6 (54/397) 10.6-17.3	94.4 (85/90) 87.6-97.6	—	—	1	1
PAX1^m or LBC ASC-US	93.2 (55/59) 83.8-97.3	24.1 (103/428) 20.2-28.3	14.5 (55/380) 11.3-18.4	96.3 (103/107) 9.08-98.6	—	—	1.01 (0.38-2.71)	1.21 (1.17-1.26)
GYPC^m or LBC ASC-US	91.5 (54/59) 81.6-96.3	25.5 (109/428) 21.6-29.8	14.5 (54/373) 11.3-18.4	95.6 (109/144) 90.1-98.1	—	—	1.00 (0.38-2.61)	1.28 (1.24-1.33)
HPV 16/18 or *PAX1*^{m}	93.2 (55/59) 83.8-97.3	67.1 (287/428) 62.5-71.3	28.1 (55/196) 22.2-34.7	98.6 (287/291) 96.5-99.5	—	—	1.02 (0.38-2.71)	3.38 (2.96-3.86)
HPV 16/18 or *GYPC*^{m}	93.2 (55/59) 83.8-97.3	72.9 (312/428) 68.5-76.9	32.2 (55/171) 25.6-39.5	98.7 (312/316) 96.8-99.5	—	—	1.02 (0.38-2.71)	3.67 (3.15-4.27)
PAX1^m or *GYPC*^{m}	88.1 (52/59) 77.5-94.1	73.6 (315/428) 69.2-77.6	31.5 (52/165) 24.9-39.0	97.8 (315/322) 95.6-98.9			0.96 (0.38-2.46)	3.71 (3.16-4.35)
For detecting CIN3+								
LBC ASC-US	89.7 (26/29) 73.6-96.4	27.7 (127/458) 23.8-32.0	7.3 (26/357) 5.0-10.5	97.7 (127/130) 93.4-99.2	1	1	—	—
HPV16/18	37.9 (11/29) 22.7-56.0	83.4 (382/458) 79.7-86.5	12.6 (11/87) 7.2-21.2	95.5 (382/400) 93.0-97.1	0.42 (0.13-1.35)	3.00 (2.40-3.78)	—	—
PAX1^m	93.1 (27/29) 78.0-98.1	76.0 (348/458) 71.9-79.7	19.7 (27/137) 13.9-27.2	99.4 (348/350) 97.9-99.8	1.04 (0.26-4.15)	2.74 (2.32-3.24)	—	—
GYPC^m	89.7 (26/29) 73.6-96.4	82.7 (379/458) 79.0-85.9	24.8 (26/105) 17.5-33.8	99.2 (379/382) 97.7-99.7	1.00 (0.40-2.52)	2.98 (2.43-3.66)	—	—
HPV 16/18 or LBC ASC-US	96.6 (28/29) 82.8-99.4	19.4 (89/458) 16.1-23.3	7.1 (28/397) 4.9-10.0	98.9 (89/90) 94.0-99.8	—	—	1	1
PAX1^m or LBC ASC-US	96.6 (28/29) 82.8-99.4	23.1 (106/458) 19.5-27.2	7.4 (28/380) 5.1-10.5	99.1 (106/107) 94.9-99.8	—	—	1.00 (0.37-2.67)	1.19 (1.14-1.23)
GYPC^m or LBC ASC-US	93.1 (27/29) 78.0-98.1	24.5 (112/458) 20.7-28.6	7.2 (27/373) 5.0-10.3	98.2 (112/114) 93.8-99.5	—	—	0.96 (0.24-3.86)	1.26 (1.21-1.30)
HPV 16/18 or *PAX1*^{m}	96.6 (28/29) 82.8-99.4	63.3 (290/458) 58.5-67.6	14.3 (28/196) 10.1-19.9	99.7 (290/291) 98.1-99.9	—	—	1.00 (0.37-2.67)	3.26 (2.89-3.67)
HPV 16/18 or *GYPC*^{m}	96.6 (28/29) 82.8-99.4	68.8 (315/458) 64.4-72.9	16.4 (28/171) 11.6-22.7	99.7 (315/316) 98.2-99.9	—	—	1.00 (0.37-2.67)	3.54 (3.10-4.05)
PAX1^m or *GYPC*^{m}	93.1 (27/29) 78.0-98.1	69.9 (320/458) 65.5-93.9	16.4 (27/165) 11.5-22.8	99.4 (320/322) 97.8-99.8			0.96 (0.31-2.99)	3.60 (3.12-4.14)

Abbreviation: CIN2+, Cervical intraepithelial neoplasia grade 2 or worse, CIN3+ Cervical intraepithelial neoplasia grade 3 or worse, *hrHPV* high-risk Human papillomavirus, *PPV* Positive predictive value, *NPV* Negative predictive value, *LBC* Liquid based cytology, *ASC-US* Atypical squamous cells of undetermined significance, *CI* Confidence interval, *HPV* Human papillomavirus, *PAX1*^{m} *PAX1* methylation, *GYPC*^{m} *GYPC* methylation

Table 4). Currently, the World Health Organization and various countries recommend hrHPV testing as the primary tool for CC screening [5, 30]. Most hrHPV-positive cases are transient infections typically clear within 2 years [31]. Therefore, triage for patients with hrHPV-positive status is essential, primarily cytological triage for patients with non-16/18 hrHPV-positive status [32]. However, there are a number of other methods that are being explored, such as double staining (p16/Ki67) [33], HPV E6/E7 mRNA [34] and DNA ploidy [35]. For the p16/Ki67 triage of hrHPV-positive women with CIN2+ and CIN3+, the sensitivities were recorded at 86.5% and 89.5%, respectively, while the specificities were noted to be 57.5% and 54.0%. Furthermore, the positive predictive values (PPVs) were determined to be 24.4% and 10.9% [33], respectively. Comparing our '*PAX1*^{m} or *GYPC*^{m}' data in CIN2+ and CIN3+, the sensitivities were 88.1% and 93.1%, while the specificities were 73.6% and 69.9%, respectively. Additionally, the positive predictive values (PPV) were recorded at 31.5% and 16.4%. Overall, based on the initial literature data regarding p16/Ki67 and our methylation study findings, the outcomes of methylation analysis demonstrated superiority over those of P16/Ki67. However, future comparative studies involving p16/Ki67 or other testing and 'PAX1m or GYPCm' within the same cohort are needed. World Health Organization Recommendations, patients who are HPV 16/18 positive or who have cytology results of ASC-US+ are recommended for referral to colposcopy [5, 36]. We also analyzed and compared the clinical performance of HPV 16/18 combined with cytology and HPV 16/18 combined with DNA methylation. The results showed no significant difference in sensitivity for detecting CIN2+ and CIN3+ between the two approaches. However, the specificity of combining HPV 16/18 with cytology for detecting CIN2+ and CIN3+ was 19.9% (95% CI: 16.3%–23.9%) and 19.4% (95% CI: 16.1%–23.3%), respectively. This indicates that the screening strategy of hrHPV initial screening and HPV 16/18 combined with cytology triage for cervical (pre)cancer still resulted in a large number of women being misdiagnosed with high-grade cervical lesions, resulting in unnecessary referrals to colposcopy and a waste of medical resources. The specificities of combining HPV 16/18 with *PAX1*^{m} and *GYPC*^{m} for detecting CIN2+ and CIN3+ exceeded 60% (Table 2). In a real-world population of individuals with hrHPV-positive status, the sensitivity and specificity of HPV 16/18 combined with DNA methylation for detecting CIN2+ were 85.9% and 60.7%, respectively [10], aligning closely with our findings.

A meta-analysis of 11 articles found that 819 out of 1,470 patients with CIN2 experienced natural regression within 2 years, with a regression rate of approximately 50% (95% CI: 43%–57%) [37]. We categorized *PAX1*^{m} and *GYPC*^{m} into three levels. The high levels of *PAX1*^{m} and *GYPC*^{m} had ORs of 113.49 (95% CI: 32.14–722.80) and 61.30 (95% CI: 19.64–270.53) for CIN3+ compared to low levels, respectively (Table 3). This may indicate a positive correlation between the level of DNA methylation and the severity of cervical lesions, as previous studies have reported that the methylation levels of various genes increase with the severity of cervical lesions [19, 20, 38].

We also evaluated *PAX1*^{m} and *GYPC*^{m} combined with cytology as a triage tool for women with hrHPV-positive status. This combination demonstrated the same sensitivity as HPV 16/18 combined with cytology but with higher specificity. The relative specificity for detecting CIN2+ was 1.21 (95% CI: 1.17–1.26) and 1.28 (95% CI: 1.24–1.33), respectively, while the specificity for detecting CIN3+ was 1.19 (95% CI: 1.14–1.23) and 1.26 (95% CI: 1.21–1.30), respectively (Table 2). This approach is also a relatively optimal strategy for triaging non-type hrHPV-positive cases. We had further insight (Supplementary Table 5) and found that, the sensitivity of *PAX1*^{m} in detecting CIN2+ and CIN3+ combined with *GYPC*^{m} for "and" was slightly less than that of HPV16/18 combined with cytology ASC-US+, while its specificity was much higher.

This study has several limitations. First, some women with hrHPV-positive status did not complete the entire study, including cytology, colposcopy, or biopsy. This led to the exclusion of their data, which may have introduced bias in the clinical performance indicators. Second, during the analysis, when calculating the relative sensitivity, there were fewer cases of CIN3+, although the sensitivity for detecting CIN3+ was consistent. Finally, as a cross-sectional study, we could not assess the future risk of developing CIN2+ and CIN3+ in women with different DNA methylation and cytology results in the future. Notably, the quality of cytology varies widely in China, especially in the underdeveloped north-west. The advantages offered by DNA methylation analysis are molecularly based, demonstrating robustness and reproducibility. Another advantage of DNA methylation analysis over cytology is sample compatibility, with both self-sampling and HPV residual specimens. Although current methylation technologies may not be suitable for large-scale implementation, technological advances and continued development of methylation analysis are expected to lead to automated and user-friendly analyses suitable for high-throughput testing in laboratories with PCR facilities. Therefore, future real-world studies with larger sample sizes are necessary for tracking and follow-up to provide more reliable evidence-based medical data and to guide the clinical application of DNA methylation.

Table 3 ORs of *PAX1* and *GYPC* methylation levels for CIN2+ and CIN3+ in hrHPV-positive women

Methylation level	OR (95%CI)[#]		
	Low	Moderate	High
PAX1^m n (ΔCp range)	347 (11.01-22.57)	77 (9.08-10.99)	63 (2.8-8.99)
CIN2+ vs. <CIN2	1	7.25 (2.69-20.61)	97.14 (41.24-261.33)
P	—	<0.001	<0.001
CIN3+ vs. <CIN3	1	4.60 (0.54-38.83)	113.49 (32.14-722.80)
P	—	0.130	<0.001
PAX1^m adjusted by HPV16/18 and LBC results			
CIN2+ vs. <CIN2	1	7.61 (2.78-21.94)	59.97 (22.91-178.20)
P	—	<0.001	<0.001
CIN3+ vs. <CIN3	1	4.41 (0.51-37.93)	57.66 (13.57-409.12)
P	—	0.147	<0.001
GYPC^m n (ΔCp range)	381 (6.01-24.71)	51 (4.01-6.00)	55 (0.37-3.94)
CIN2+ vs. <CIN2	1	21.31 (8.75-55.94)	81.59 (35.11-211.49)
P	—	<0.001	<0.001
CIN3+ vs. <CIN3	1	23.44 (6.51-110.10)	61.30 (19.64-270.53)
P	—	<0.001	<0.001
GYPC^m adjusted by HPV16/18 and LBC results			
CIN2+ vs. <CIN2	1	16.70 (6.44-46.09)	45.95 (18.52-125.05)
P	—	<0.001	<0.001
CIN3+ vs. <CIN3	1	13.42 (3.28-6.82)	23.87 (6.49-115.42)
P	—	<0.001	<0.001

Abbreviation: OR Odds ration, *CIN2* Cervical intraepithelial neoplasia grade 2, *CIN2+* Cervical intraepithelial neoplasia grade 2 or worse, *CIN3* Cervical intraepithelial neoplasia grade 3, *CIN3+* Cervical intraepithelial neoplasia grade 3 or worse, *PAX1*^{m} *PAX1* methylation, *GYPC*^{m} *GYPC* methylation, *HPV* Human papillomavirus, *LBC* Liquid based cytology

[#] OR and 95%CI were calculated using logistic regression

Conclusion

High levels of *PAX1*^{m} and *GYPC*^{m} were effective indicators for identifying CIN3+ (all ORs > 20). The sensitivities of *PAX1*^{m}, *GYPC*^{m}, and cytology for detecting CIN2+ and CIN3+ were comparable. Compared to the hrHPV-positive triage strategy (HPV 16/18 combined with cytology), the specificities of HPV 16/18 combined with *PAX1*^{m} and *GYPC*^{m} were three times higher. Therefore, DNA methylation may serve as an essential tool for detecting cervical (pre)cancer in women with hrHPV-positive status.

Abbreviations

AGC	Atypical glandular cells
ASC-H	Atypical squamous cells cannot exclude HSIL
ASC-US	Atypical squamous cells of undetermined significance
AUC	Area under the curve
CC	Cervical cancer
CIN1	Cervical intraepithelial neoplasia grade 1
CIN2	Cervical intraepithelial neoplasia grade 2
CIN3	Cervical intraepithelial neoplasia grade 3
CIN2+	Cervical intraepithelial neoplasia grade 2 or worse
CIN3+	Cervical intraepithelial neoplasia grade 3 or worse
CI	Confidence interval
DNA	DeoxyriboNucleic Acid
GYPC^m	*GYPC* methylation
HPV	Human papillomavirus
hrHPV	high-risk Human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IQR	Interquartile range
LBC	Liquid based cytology
LSIL	Low-grade squamous intraepithelial lesion
NILM	No intraepithelial lesions or malignancy
NPV	Negative predictive value
OR	Odds ration
PAX1^m	*PAX1* methylation
PPV	Positive predictive value
ROC	Receiver operating characteristic curve

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-13126-4.

Supplementary Material 1.

Acknowledgments

Not applicable.

Authors' contributions

Hong Tao carried out the experiment of the study, read the relevant literatures, performed the statistical analyses, interpreted the results and drafted the manuscript. Fang Yu, Li Yang, Xiaozhu Pei and Saiping Mao were involved in the design of the study, participated to collect specimens, do the experiment and helped to revise the manuscript. Xing Fan were involved in the

conception and design of the study and direct the progress of the study. All authors read and approved the final manuscript.

Funding
This work was supported by the Research Project of department of science and technology of Changsha (kh2302012).

Data availability
The datasets used during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This research project was approved by the Research and Clinical Trial Ethics Committee of the Changsha Hospital for Maternal & Child Health Care (No. EC-20230726-02), informed consent was obtained from all patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 25 September 2024 Accepted: 29 October 2024
Published online: 04 November 2024

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